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(54) Title: THERAPEUTIC PEPTIDES AND PROTEINS

(57) Abstract

Liposomes are disclosed which prolong the bioavailability of therapeutic peptides or proteins. The liposomes are unilamellar or oligolamellar vesicles which are stable in lymph, but unstable in serum or plasma. The net electric charge of the lipid vesicle is negative or neutral and the vesicle contains less than 10 mole percent cholesterol. Bioavailability of the therapeutic peptides or proteins is enhanced by combination of the liposomes with an isotonic carrier solution having an osmolarity of between about 200 to 400 mosmol. Bioavailability is further enhanced by subcutaneous injection of the liposomes. The addition of empty liposome to liposome encapsulated therapeutic agents was found to increase bioavailability. Further, the use of empty liposomes as a diluent for solutions of free peptides end proteins increases shelf life.

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"THERAPEUTIC PEPTIDES AND PROTEINS"

BACKGROUND OF THE INVENTION

1. Field of the Invention.

The present invention relates generally to drug delivery systems in which a therapeutic peptide or protein is protected by encapsulation in microspheres. More particularly, the present invention relates to those drug delivery systems in which the therapeutic protein or peptide is encapsulated in fat soluble spheres known as liposomes.

2. Description of Related Art.

Liposomes are tiny spheres which are made from natural and/or synthetic lipids. Liposomes have been used to encapsulate various drugs in order to protect them from degradation by serum proteases and removal by the kidney and other cells or organs during drug treatment. Although liposomes have been found to provide good protection during parenteral introduction into animals or patients, there have been substantial problems in achieving the desired rates of drug release once the liposomes have been administered.

It is readily apparent that many therapeutic peptides and proteins cannot exert their desired effects if retained within the liposome. Furthermore, attempts to design liposomes which can provide sustained release of biological activity have been generally unsuccessful. The liposome must be sufficiently stable to contain the peptide or protein and protect it from enzymatic degradation until releasing the agent into the circulation. On the other hand, the liposome must also be sufficiently unstable to provide reproducible rates of peptide or protein release. Accordingly, there is a

present need to provide liposome carriers which provide protection of therapeutic peptides and proteins during parenteral administration while not providing such a high degree of protection that appropriate release and utilization of the agent in vivo is precluded.

An example of the type of problems facing researchers investigating liposome-based delivery systems is the complex nature of the lymphatic and blood systems. When the particular drug is targeted to reach the blood stream, the liposome must be resistant to attack in the lymphatic system, while at the same time being readily vulnerable to gradual dissolution in the blood stream. However, the stability of the liposome in the blood stream must not be so great that the liposomes which carry the peptide or protein are taken up and destroyed by macrophages prior to release of the therapeutic agent.

The competing considerations present in the complex human body have required continuing research into the design and development of liposomes which provide the desired degree of protection, while at the same time providing controllable and reproducible release of the entrapped therapeutic peptide or protein. Accordingly, there is still a need to provide liposomes which increase or prolong the bioavailability of therapeutic peptides and proteins. In addition to the need for liposomes which enhance bioavailability of therapeutic peptides and proteins, there is also a need for additives or diluents which can be added to the liposome entrapped therapeutic materials to provide increased and prolonged biological effects.

Another important consideration in any drug delivery system is the stability of therapeutic peptides and proteins during storage prior to use in the body. Oxidation and hydrolysis, which can result in the loss of chemical integrity of therapeutic agents, can

frequently be controlled by adjusting the acidity of the excipients, by controlling the temperature of the storage conditions, by manufacturing and storage in an inert atmosphere, or by the addition of anti-oxidants. Physical stability is typically controlled by selection of a suitable solubilizing solvent, surfactant or surfactant system, or by adjusting the pH. For therapeutic agents that can not withstand storage in an aqueous environment, lyophilization is frequently used. A lyophilized product is not the most preferred dosage form because it is expensive to develop and manufacture and because it requires the customer to hydrate the material with a diluent immediately prior to use.

Peptides and recombinant proteins are particularly notable for difficulties in pharmaceutical formulation and manufacturing. In addition to the usual chemical oxidation and hydrolysis problems commonly associated with conventional therapeutic agents, the formulator must be concerned about affects that are broadly categorized as "denaturation". For therapeutically active polypeptides, the 3-dimensional organization (secondary and tertiary structure) of the peptide polymer is usually regarded to be essential for activity. It is therefore, generally felt that maintenance of this 3-dimensional geometry in a pharmaceutical formulation must be one of the critical determinants of the integrity and shelf-life of the product.

Another stability determinant which is somewhat unique to polypeptides is aggregation. Proteins are particularly subject to aggregation problems because of their large size and tendency to hydrogen bond with other peptides and to associate through hydrophobic interactions. These same properties result in the tendency for proteins and peptides to accumulate at an air/water interface, and to interact with the surface of

containers, delivery tubing and syringe barrels - all factors which affect the stability of the final product.

Because of the commonality of this problem in pharmaceutical formulation and because of the surge of biotechnology activity related to protein formulation, it would be a useful addition to the formulary to have a pharmaceutically acceptable (aqueous) diluent that would overcome the general problem of adsorption of therapeutically active agents, including polypeptides, to surfaces and to the air water interface.

SUMMARY OF THE INVENTION

In accordance with the present invention, a method and composition is provided for prolonging the bioavailability of therapeutic peptides and/or proteins when administered to an individual. The invention is based on the discovery that the bioavailability of therapeutic peptides or proteins can be prolonged in the body when encapsulated in liposomes having certain specific characteristics. The liposome must be unilamellar or oligolamellar. Further, the liposome must be composed of suitable lipids such that it is relatively stable in lymph, but releases its contents readily in serum or plasma. Further, the overall net electric charge of the lipid vesicle must be negative or neutral and the liposome must contain less than 10 mole percent of cholesterol.

It was discovered in accordance with the present invention that liposome vesicles having the above described properties are effective in prolonging the bioavailability of therapeutic peptides or proteins encapsulated therein. The liposomes of the present invention are believed to prolong bioavailability by providing a vesicle which has sufficient structural integrity and characteristics which resist dissolution at the point of injection and in lymphatic system, while

at the same time being amenable to gradual release of the encapsulated peptide or protein in the blood stream. Further, the lipid vesicles containing the therapeutic peptide or protein are not so stable that they are taken up and consumed by macrophages prior to release of the therapeutic agent.

As another feature of the present invention, it was discovered that the bioavailability of therapeutic peptides or proteins is further enhanced when the liposomes of the present invention are disbursed in a pharmaceutically acceptable isotonic carrier solution wherein the osmolarity of the carrier solution is between about 200-400 mosmol.

As an additional feature of the present invention, it was discovered that the increased bioavailability of the therapeutic peptide or protein is further enhanced when the peptide or protein is encapsulated in accordance with the present invention, dispersed in the isotonic carrier described above and subcutaneously injected into the patient. Such subcutaneous injection provides increased bioavailability and biological effectiveness over administration procedures involving oral administration, intravenous injection, inhalation and the like. It is believed that the subcutaneous injection of compositions in accordance with the present invention results in liposome interaction with the lymph prior to entry into the blood stream where the therapeutic peptides or proteins become bioavailable. However, the complex biological mechanisms and interactions which produce the improved results via subcutaneous injection is novel and unexpected and is not entirely understood by applicant.

A further aspect of the present invention involves the discovery that the bioavailability of encapsulated therapeutic peptides and proteins is increased markedly when empty liposomes are added to the liposome encapsu-

lated therapeutic agent prior to administration. Empty liposomes were also found to be useful as a diluent to solubilize polypeptides and/or proteins to improve stability and prevent adsorption of these therapeutically active agents to containers.

The above-discussed and many other features and attendant advantages of the present invention will become apparent as the invention becomes better understood by a reference to the following detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1-17 are graphs and charts depicting results of the examples.

DETAILED DESCRIPTION OF THE INVENTION

The liposomes of the present invention are useful in encapsulating a wide variety of therapeutic peptides and proteins. The peptide or protein should have a molecular weight of below 250,000 with peptides and proteins having molecular weights within the range of 500 - 100,000 being preferred. The following peptides or proteins, their analogs, related peptides, fragments, inhibitors and antagonists may be encapsulated: Transforming growth factors (TGF-alpha and TGF-beta); Interleukins (IL-1, IL-2, IL-3); Interferons (IFN-alpha, -beta and -gamma); Calcitonins (salmon, human, avian, synthetic, etc.); Insulin-like growth factors (IGF-1, also called somatomedin C; IGF-2, also called multiplication-stimulating activity); Parathyroid hormone (PTH, native 1-84 and 1-34 fragments, human, bovine sequences, synthetic analogues and antagonists); Granulocyte-macrophage stimulating factor (GMCSF); Macrophage-stimulating factor (MCSF); Erythropoietin; Insulins; Amylins; Glucagons; Lipocortins; Growth hormones; Somatostatin (analogues and antagonists);

Gonadotropin releasing hormone (GNRH); Luteinizing hormone releasing hormone (LHRH); Platelet-derived growth factor; Thromboplastin activators; tissue plasminogen activators; Streptokinase; Vasopressin; Muramyldipeptide (MDP); Atrial naturetic factor (ANF); Calcitonin gene-related peptide (CGRP); Bombesin; Enkephalins; Vasoactive intestinal peptide (VIP); Epidermal growth factor (EGF); Fibroblast growth factor (FGF); Growth hormone releasing hormone (GRH); Peptide T and peptide T amide; Herpes virus inhibitor; Virus replication inhibiting factor; and soluble CD_4.

The following peptides, their analogs, related peptides, fragments, inhibitors and antagonists thereof may also be included: ACTH and fragments; angiotensins; angiotensin converting enzyme inhibitors (ACE inhibitors); bradykinin (BK), BK analogs and BK antagonists; C-peptides; pro-insulin; calcitonin C-terminal adjacent peptide; human calcitonin precursor peptide (katacalcin) and analogs; hypercalcemia malignancy factor (PTH like adenylate cyclase-stimulating protein); beta casomorphins; chemotactic peptides and inhibitors; corticotropin releasing factors (CRF) and related peptides; dynorphins; endorphins; fibrinopeptides and analogs; fibronectin fragments and related peptides; caerulein; cholecystokinins, fragments and analogs; galanin; gastric inhibitory polypeptide (GIP); gastrins; gastrin releasing peptide (GRP); motilin; pancreatic polypeptides; PHI peptides; PHM peptides; peptide YY; secretins; melanocyte stimulating hormones (MSH); neuropeptide Y (NPY); neuromedins; neuropeptide K; neurotensins; phosphate acceptor peptides (c-AMP protein kinase substrates); oxytocins; substance P; TRH; substance P antagonists; and enkephalins.

The liposome vesicles of the present invention can be made by any of the conventional processes known for preparing liposomes including thin film shaking or

sonification (A.D. Bangham, M.M. Standish, and J.C. Watkins, J. Mol. Biol., 13, 238 (1965)); detergent removal (Y. Kagawa and E. Racker, J. Biol. Chem., 246, 5477 (1971).; J. Brunner, P. Skrabal, and H. Hauser, Biochem. Biophys. Acta, 455, 322 (1976); reverse-phase evaporation (F. Szoka and D. Papahadjopoulos, Proc. Natl. Acad. Sci. USA, 75, 4194 (1978)); ether injection methods (D. Deamer and A.D. Bangham, Biochim. Biophys. Acta, 433, 629 (1976)); freeze-drying, (J.R. Evans, F.J.T. Fildes and Jean Oliver, U.S. 4,311,712, 1982 and U.S. 4,370,347, 1983 and K. Harada, H. Miura, and T. Ohsawa, Japan Kokai, 82310 (1982); Idem, ibid., 82311 (1982)); use of microfluidization (Mayhew, et al. 1984); extrusion (C.A. Hunt and D.P. Papahadjopoulous, U.S. 4,529,461, 1985, L.D. Mayer et al. Biochim. Biophys. Aceta, 858:161, 1986 and F.J. Martin and J.K. Morano, U.S. 4,737,323, 1988); formation of multivesicular liposomes (S. Kim, M. Turker, E. Chi, et al., Biochim. Biophys. Acta, 728:339, 1978) and other known conventional liposome preparation procedures. The conditions of the procedure must be such that the resulting liposome is unilamellar or oligolamellar and has an outside diameter of between about 10 - 10,000 nanometers. The preferred diameter size range is from between about 20 - 200 nanometers.

The lipids used in preparing the liposomes of the present invention can be made from any of the conventional synthetic or natural phospholipids used in liposome formulation and include phospholipids from natural sources such as eggs, plants and animal sources such as (but not limited to) phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid, phosphatidylglycerol, phosphatidylserine, phosphatidylinositol and sphingomyelin. Synthetic phospholipids may also be used such as (but not limited to) dimyristoyl-, dipalmitoyl-, distearoyl- and dioleoyl- analogues of

phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidic acid and phosphatidylserine. Other additives such as cholesterol and other sterols, glycolipids, cerebrosides, gangliosides, sphingosines, glucopsychosine, or psychosine may be added as conventionally known. The relative amounts of phospholipids and additives used in the liposomes may be varied if desired. The preferred ranges are 80-100 percent phospholipid and 0 - 20 percent of the additives noted above. Cholesterol must comprise less than 10 mole percent if used in the preparations. The compositions which are effective in this invention will be relatively stable in buffer or in lymph fluid and reasonably unstable in serum or plasma in order to provide for release of the therapeutic peptide or protein into the circulation so that it may have its desired biological effect. The relative stability is determined by measuring the rate of leakage of the therapeutic agent between 1 to 24 hours during incubation in 90 volume percent serum and 20 volume percent serum at 37°C. Phosphate buffered saline (PBS) is used to dilute the serum with the 20 volume percent saline being considered equivalent to lymph fluid.

In accordance with the present invention, the liposome containing the encapsulated peptide or protein should leak more than 15 percent of the peptide or protein during the above incubation in 90 volume percent serum. Conversely, the liposome should leak less than 15 percent of the peptide or protein during the above incubation in 20 volume percent serum.

Unilamellar and oligolamellar liposomes have an inner volume wherein the therapeutic peptide or protein is entrapped. Multilamellar liposomes are not preferred for use in accordance with the present invention. In preparing liposomes, the resulting vesicle mixture may include varying amounts of unilamellar, oligolamellar

and multilamellar vesicles. Although it is preferred that the liposomes used in accordance with the present invention be unilamellar or oligolamellar, effective compositions can be prepared wherein up to 25% by weight of the liposomes are of the multilamellar type. Greater amounts of multilamellar liposomes may result in reduced effectiveness of the composition.

The particular lipids and any additives present in the liposome must be such that the net electric charge of the liposome is negative or neutral. Negative and neutral liposomes may be identified and separated by electrophoresis or other separation techniques based on differential charge migration. Up to 99 percent of the lipid may be negatively charged, the remainder consisting of a neutral lipid component.

Although any of the normal administration procedures may be used to introduce the encapsulated peptide or protein into the patient, it was discovered that subcutaneous injection provided an increased effect for the encapsulated calcitonin which was over and above other injection or administration regimens. In addition, it was found that isotonic carrier solutions having an osmolarity of between about 200 to 400 mosmol provided increased biological effect for the encapsulated calcitonin. Examples of such isotonic carrier solutions are 0.9% saline, 5% sorbitol, 5% dextrose, 9.25% sucrose and 290 mOsm phosphate buffers (PBS).

Examples of practice are as follows:

Example 1

Egg phosphatidylcholine (egg PC) and egg phosphatidylglycerol (egg PG) were obtained from Avanti Polar Lipids (Birmingham, Alabama). 110 mg of egg PC and 46.4 mg of egg PG were added to a 50 ml round bottom flask and the solvents were removed in vacuo in a rotary evaporator at 37 degrees centigrade leaving a thin film of lipid on the flask. Diethylether (2 ml) was added

and the evaporation in vacuo was repeated two times and the flask containing the thin lipid film was placed under vacuum (0.005 mm mercury) at room temperature overnight to remove traces of water and other solvents. 0.168 mg (25.2 MRC units) of human calcitonin (hCT), (source: Bachem, Torrance, California), and 6,150 CPM of 125-I-labeled hCT tracer (Incstar, Minneapolis, Minnesota) were dissolved in 1.0 ml of phosphate buffered saline, pH 7.4. This mixture was added to the flask containing the phospholipid thin film and the flask was agitated gently on a wrist shaker for 5 minutes or until all of the lipid film was dispersed.

The suspension was frozen by immersion in a methanoly-dry ice mixture and thawed quickly in a water bath adjusted to 37 degrees centigrade. The suspension was transferred to a Lipex Extruder (Lipex Biomembranes, Vancouver, British Columbia, Canada) containing two stacked polycarbonate filter discs of 0.2 micron pore diameter (Nucleopore, part number 110606, Pleasanton, California). The freeze/thaw step was repeated and the resulting suspension was subjected to extrusion as before. This process was repeated a total of five times. After the last extrusion, the extruder cell and filters were washed with three successive 0.5 ml portions of phosphate buffered saline and the washes were combined with the original suspension.

The liposomal hCT suspension was placed on a column of Sepharose 4B (Pharmacia, Piscataway, New Jersey) having dimensions of 1 cm diameter and 20 cm height. The column was eluted with phosphate buffered saline at room temperature at a flow rate of 20 ml/hr and fractions of 1.0 ml were collected and assayed for their content of 125-I-labeled hCt. The liposomal hCT fractions eluting at the void volume of the column, representing 61% of the total counts, were combined. The calcitonin content of the liposomal fraction was calcu-

lated from the ratio of ^{125} iodine hCT tracer to hCT present in the original solution. The unentrapped hCT was recovered in the salt volume of the column; these fractions were combined, counted and the concentration of hCT determined as noted above.

Male rats of the Fisher 344 strain (Harlan, Indianapolis, Indiana) weighing approximately 150gm were fasted overnight. The rats were anesthetized with Metofane®, weighed and injected subcutaneously with the liposomes consisting of egg PC/egg PG (7/3) containing hCT or free hCT in a dosage of 1.44 units of hCT per kg body weight. Control animals were injected with an equivalent amount of empty liposomes in phosphate buffered saline. After varying periods of time the animals were anaesthetized with Metofane® and an abdominal incision was made; blood samples were taken from the inferior vena cava and the serum was analyzed for calcium by the method of Liedtke, Clinical Chemistry, 27: 2025-2028, 1981. The results of the test are shown in Table 1.

TABLE 1

Effect Of Liposomal hCT (eggPC/eggPG, 7/3) Free hCT and Empty Liposomes on the Serum Calcium of Rats

	Δ Serum Calcium, mg/dl		
Time, hr.	Empty Liposome	Liposome hCT 1.44 U/kg	Free hCT, 1.44 U/kg
0	0	0	0
1	+0.28	-1.5*	-2.4*
2	+0.27	-1.7*	-3.1*
3	+0.15	n.d.	-2.6*
4	+0.08	-0.8*	-0.4*
5	0	n.d.	0
6	+0.12	-0.7*	n.d.
8	+0.07	-0.4*	n.d.
10	+0.33	-0.4*	n.d.
12	+0.04	-0.7*	n.d.
18	+0.02	-0.2	n.d.
24	+0.25	-0.1	0

n.d. = not determined

*Results are the mean of three separate experiments. Data points which are significantly different from the zero time control ($p < 0.05$) are marked with an asterisk.

The results show that the liposomes of the present invention consisting of egg phosphatidylcholine egg phosphatidylglycerol and having a net negative surface charge provide a prolonged biological effect of calcitonin on serum calcium which is not possible with free calcitonin. Furthermore, an identical amount of liposomes of the same composition without hCT, containing only buffer are without effect on the serum calcium.

Example 2

In this example liposomes containing hCT were prepared exactly as in Example 1 except that the phospholipid consisted of 150 mg of egg PC. The egg PC liposomes containing hCT were administered to rats as detailed in Example 1 except that the dose of hCT administered was 0.36 units per kg body weight. Blood samples were obtained and analyzed as in Example 1. The results of the tests are shown in Table 2.

TABLE 2

Effect of Liposome hCT (eggPC) on the Serum Calcium of Rats

Time, hr	Serum Ca, mg/dl eggPC/hCT, 0.36 U/kg
0	0
1	-0.6*
2	-0.7*
3	-0.8*
4	-0.4
5	-0.4*
6	-0.5*
8	-0.5*
10	-0.9*
12	-0.3
18	-0.1
24	-0.1

* - $p < 0.05$ versus control. Other conditions as in Table 1.

The results show an extended bioavailability of calcitonin which results in the prolonged reduction of serum calcium. This is provided by liposomes consisting of a natural phospholipid (egg phosphatidylcholine) and having a net neutral charge in accordance with the present invention.

Example 3

In this example, hCT-containing liposomes were prepared as described in Example 1 except that the prospholipids consisted of 95 mg of dimyristoylphosphatidylcholine (DMPC) and 40 mg of dimyristoylphosphatidylglycerol (DMPG). This represents a molar ratio of DMPC/DMPG of 7/3. The hCT-containing liposomes were administered to rats according to the procedure detailed in Example 1 except that the dose of hCT was 0.36 MRC units per kg body weight. Blood samples were obtained as noted in Example 1 and the serum calcium level was measured as also noted in Example 1. The results of the tests are shown in Table 3.

TABLE 3

Effect of Liposome hCT (dimyristoylPC/dimyristoylPG, 7:3) on the Serum Calcium of Rats

	Serum Calcium, mg/dl
Time, hrs	DMPC/DMPG; 7/3; 0.36 U/kg
0	0
1	-1.1
2	-0.6
4	-0.2
6	-0.2
8	-0.1
10	-0.3
12	-0.7
18	-0.3
24	-0.3

* $p < 0.05$; other conditions as in Table 1.

These results further demonstrate the prolonged biological effect provided by liposomes consisting of

synthetic phospholipids having a net negative charge in accordance with the present invention.

Examples 4-6

Example 4 - Large multilamellar extruded liposomes: hCT containing liposomes were prepared by extrusion using 150 mg of egg phosphatidylcholine and 25.2 units of human calcitonin as described in Example 1 with the following modifications: The polycarbonate filters of 0.2 micron pore diameter were replaced with 1.0 micron pore diameter filters and the lipid/hCT suspension was frozen and thawed five times followed by only one extrusion through the filters which results in the production of multilamellar vesicles approximately one micron in diameter as previously shown by Mayer, et al., (Biochim. Biophys. Acta, 858:161, 1986. When compared with the formulations shown in Examples 2 and 3, which were tested at an equivalent dosage, one micron multi-lamellar extruded vesicles provide a more pronounced initial decline at one and two hours, but have a lesser calcium-lowering potential at 10 to 24 hours. Multi-lamellar vesicles containing hCT prolong its effect slightly but are not the preferred dosage form.

Example 5 - Positively charged liposomes (egg phosphatidylcholine/stearylamine, 9/1): hCT-containing liposomes (single lamellar vesicles) were prepared by extrusion as in Example 1 except that: (1) the lipid consisted of 176 mg of egg phosphatidylcholine and 6.7 mg of stearylamine (both obtained from Avanti Polar Lipids, Birmingham, AL); and, (2) the chromatography column used to separate entrapped and unentrapped hCT contained Sephadex G-75 instead of Sepharose 4B column dimension. The elution conditions were similar to those described in Example 1. Extended liposomes containing hCT and having a positively charged component like stearylamine do not exhibit significant calcium-lowering

effects after one hour and are not a preferred dosage form.

Example 6 - Extruded liposomes containing 10 mole percent cholesterol (DMPC/DMPG/Cholesterol, 7/3/1.1): hCT-containing oligolamellar and single lamellar liposomes were prepared with 95 mg dimyristoylphosphatidylcholine, 40 mg of dimyristoylphosphatidylglycerol and 8.6 mg of cholesterol and 25.2 units of human calcitonin by the procedure described in Example 1. As shown in Table 4, when cholesterol is present in 10 mole% in the lipid bilayer of single lamellar vesicles which are otherwise effective in prolonging the biological effect of hCT (see Table 3), the biological effect of hCT to lower serum calcium is abolished. Liposomes containing more than 10 mole% of cholesterol are therefore not a preferred dosage form.

TABLE 4

Comparison of different formulations of liposomal hCT and their effect on the serum calcium of rats following subcutaneous administration

	Change in Serum Calcium, mg/dl		
Time, hr	Example 4 1000 nm MLV egg PC; 0.36 U/kg	Example 5 200 nm SLV eggPC/SA, 9/1; 0.36 U/kg;	Example 6 200 nm SLV DMPC/DMPG/Chol 7/3/1.1 0.36 U/kg
0	0	0	0
1	-1.77*	-0.60*	-0.02
2	-1.16*	-0.10	-0.16
4	-0.45*	+0.15	+0.18
6	-0.28*	-0.05	-0.02
8	-0.34*	+0.08	-0.16
10	-0.36*	-0.25	-0.16
12	-0.10	-0.10	+0.08
18	-0.07	+0.18	+0.22
24	-0.05	+0.11	+0.30

The results represent the change in serum calcium from the zero time level and are the average of duplicate assays from three separate animals. The control calcium level at time zero was 10.2 0.2 mg/dl;

* $p < 0.05$ versus time zero

Abbreviations: PL - phosphatidylcholine; SA - stearylamine; DMPC - dimyristoylphosphatidylcholine; DMPG - dimyristoylphosphatidylglycerol; Chol - cholesterol; MLV-multilamellar vesicle; SLV - single lamellar vesicle; U - MRC units; kg - kilogram of body weight.

Example 7

The leakage rates of various encapsulated hCT was measured in accordance with the incubation procedure in 90 volume percent and 20 volume percent serum as

previously described. The liposomes tested were egg PC, egg PC/eggPG(7/3), DSPC/DPPG(9/1) and egg PC/cholesterol(2/1). The procedures used were as follows:

1. <u>Purification of human calcitonin ^{125}I-labelled tracer</u>

Synthetic human calcitonin labelled with ^{125}I (specific activity, 1.8 Ci/mg) was obtained from the Nichols Institute (San Juan Capistrano, CA). The entire sample representing 10 uCi was taken up in a small volume of phosphate buffered saline (PBS), pH 7.2, and applied to a 10 ml Biorad 10DG desalting column (Richmond, CA), 1.7 X 4.8 cm, equilibrated in the same buffer. The material was eluted at room temperature with PBS as noted above and collected in fractions of 0.5 ml. The voiding peak which represents ^{125}I-hCT was collected and used for liposome encapsulation as noted below.

2. <u>Preparation of liposomes containing ^{125}I hCT</u>

200 micromoles of lipid (as indicated above) was placed in a 100 ml round bottom flask and the solvent was removed in vacuo with a rotary evaporator. The lipid was dissolved in diethylether and the evaporation procedure was repeated twice. The thin lipid film which resulted is further dried by placing the flask under vacuum overnight in a dessicator.

^{125}I-hCT (2-10 uCi) in PBS was added to the 100 ml flask containing the thin lipid film and the mixture was shaken gently until all of the lipid film was noted to be removed from the wall of the flask. It was then frozen quickly in an acetone-dry ice mixture, thawed at 37°C in a water bath, and extruded through two stacked 200 nanometer polycarbonate filters (Nucleopore, Pleasanton, CA) in a Lipex Extruder (Lipex Biomembranes, Vancouver, BC, Canada) as described in Example 1. The resulting suspensions of ^{125}-I-hCT-containing liposomes were passed over a column of Sephadex G-75 (1 X 20 cm)

at a flow rate of 20 ml per hour and the liposome-encapsulated hCT was separated from free hCT by combining the voiding fractions and used in the leakage studies with 90% and 20% rat serum.

3. Serum-induced leakage

200 ul of the ^{125}I-hCT-containing liposomes, representing 200,000 to 1,000,000 CPM, was added to 1800 ul of rat serum or rat serum diluted with PBS in such a way as to obtain a final serum concentration of either 90% or 20%. The samples were incubated at 37° C for 1, 3, 6 and 24 hours. Leakage was measured at these times by injecting an aliquot of the sample into a HPLC gel permeation column (TSK-3000SW, 7.5mm X 30 cm, Beckman Instruments, Fullerton, CA) and a SW-precolumn (7.5 mm x 7.5 cm, Beckman) at a flow rate of 1.0 ml per minute. Fractions of 0.250 ml were collected and counted using a Searle Analytical Gamma Counter. The activity present in the void peak represents the ^{125}I-labelled hCT remaining in liposomes. The counts in the combined void fractions were summated and the leakage between 1 and 24 hours was determined.

The results of the leakage tests are shown in Table 5.

TABLE 5

SUMMARY OF LEAKAGE RATES

	% Loss of hCT from Liposome peak, 1-24 hours at 37 C°	
Liposome type	90% serum	20% serum
egg PC	30	7.6
ePC/ePG (7/3)	21.6	0
DSPC/DPPG (9/1)	0	4.4
ePC/chol (2/1)	5.7	3.5

The addition of empty liposomes was found to enhance the bioavailability of hCT which was encapsulated in liposomes. This phenomenon is not limited to the specific types of liposomes described previously, but is applicable to liposome encapsulated therapeutic agents in general. Preferably, the amount of empty liposomes added to the encapsulated peptide or protein will be sufficient to provide a weight ratio of encapsulated liposomes to empty liposomes of between about 1:1 and 1:10,000. The empty liposome should have a composition that is the same or similar to the liposome in which the therapeutic peptide or protein is encapsulated. However, empty liposomes having substantially different compositions may be used. The size and character of the liposome may be varied with the optimum

enhancement being determined experimentally. The optimum ratio of loaded liposomes to empty liposomes can also be determined experimentally.

Although the following examples are limited to the use of specific empty liposomes in combination with encapsulated hCT to enhance bioavailability, it will be understood by those skilled in the art that the present invention has wide application to the addition of empty liposomes to a variety of encapsulated peptides or proteins to increase their bioavailability. The peptides, proteins and liposome forming materials set forth previously are examples of the type of therapeutic agents and liposomes which may be used in accordance with this aspect of the invention.

The encapsulated therapeutic agent, once the empty liposomes have been added, is administered to the patient by any of the well known methods commonly used for liposome encapsulated peptides and proteins. The only difference being that the dosage per unit volume will be decreased slightly depending upon the amount of empty liposomes which have been admixed. The same pharmaceutically acceptable diluents and carriers may be used. The following example demonstrates the marked increase in bioavailability resulting from addition of empty liposomes to hCT encapsulated in liposomes.

<u>Example 8</u>

Four formulations were manufactured in a manner similar to Example 1 except that a higher concentration of HCT in the hydration buffer was used and other conditions about the formulation were modified as according to Table 6. When the higher concentration of HCT is used, the product manufactured at pH 7.4 can not be extruded due to precipitation of the HCT. This problem can be overcome by manufacturing at pH 5.0. At pH 5.0 HCT is much more soluble and there are no problems with the extrusion.

TABLE 6

Formulation Code	Buffer & Conc.	pH	Isotonocifer	Osmolarity (mOsm/kg)	Drug	[HCT] in hydration buffer (ug/ml)	Lipid composition	[Lipid] in hydration buffer (mM)	Alpha-tocopherol (%)
A	Phosphate, 20 mM	7.40	Sorbitol	290	HCT	500	POPC/DOPG (70/30)	100	0.15
B	Phosphate, 20 mM	7.40	NaCl	290	HCT	500	POPC/DOPG (70/30)	100	0.15
C	Acetate, 20 mM	5.00	Sorbitol	290	HCT	500	POPC/DOPG (70/30)	100	0.15
D	Acetate, 20 mM	5.00	NaCl	290	HCT	500	POPC/DOPG (70/30)	100	0.15

	Manufacturing	Comments
A	Extrusion (200 nm)	Can't be extruded (due to the precipitation of HCT due to higher HCT concentration in hydration buffer)
B	Extrusion (200 nm)	Can't be extruded (due to the precipitation of HCT due to higher HCT concentration of hydration buffer)
C	Extrusion (200 nm)	extruded nicely
D	Extrusion (200 nm)	extruded nicely

Example 9

Six additional formulations were manufactured with the compositions indicated on Table 7. The percent capture and HCT/lipid ratio in the final product is shown on this table. The data indicate that the % capture and corresponding HCT/lipid ratio increases as the mole of PG increases. Up to 75% of the drug can be captured under optimal conditions. The peptide solubility appears to be lower under higher ionic strengths.

TABLE 7

Formulation Code	Buffer & Conc.	pH	Isotonocifer	Osmolarity (mOsm/kg)	Drug	[HCT] in hydration buffer (ug/ml)	Lipid composition	[Lipid] in hydration buffer (mM)	Alpha-tocopherol (%)
A	Acetate, 20 mM	5.00	Sorbitol	290	HCT	500	POPC/DOPG (100/0)	100	0.15
B	Acetate, 20 mM	5.00	Sorbitol	290	HCT	500	POPC/DOPG (90/10)	100	0.15
C	Acetate, 20 mM	5.00	Sorbitol	290	HCT	500	POPC/DOPG (70/30)	100	0.15
D	Acetate, 20 mM	5.00	NaCl	290	HCT	500	POPC/DOPG (100/0)	100	0.15
E	Acetate, 20 mM	5.00	NaCl	290	HCT	500	POPC/DOPG (90/10)	100	0.15
F	Acetate, 20 mM	5.00	NaCl	290	HCT	500	POPC/DOPG (70/30)	100	0.15

	Manufacturing	Comments	Stability	Drug/Lipid	% Capture
A	Extrusion (200 nm)	extruded nicely	Stable	0.00078	35%
B	Extrusion (200 nm)	extruded nicely	Stable	0.00787	52%
C	Extrusion (200 nm)	extruded nicely	Stable	0.00763	74%
D	Extrusion (200 nm)	extruded nicely	Sedimentation	0.00084	38%
E	Extrusion (200 nm)	extruded nicely	Stable	0.00133	60%
F	Extrusion (200 nm)	extruded nicely	Stable	0.00166	75%

Example 10

A procedure was developed that can remove HCT that is absorbed to the surface of lipid vesicles. The procedure involves exposing the formulation to suspensions of silica or polyacrylamide (SM-7) beads. These beads remove unencapsulated HCT from the formulation. The data on Fig. 15 show that preparations manufactured with 30 mole % PG at low ionic strength (sorbitol) have a significant fraction of their peptide accessible to extraction by the bead treatment. This suggests that this extractable fraction is associated loosely on the surface of the vesicles, and is not actually encapsulated in the internal volume of the vesicles. The fraction of extractable peptide is much less significant when the formulations are manufactured with 10 and 0 mole % PG (See Figs. 16 and 17, respectively). Formulations manufactured at high ionic strength do not have an extractable fraction of the peptide.

Example 11

In this example, human calcitonin was encapsulated in 200 nanometer single lamellar extruded vesicles consisting of 1-palmitoyl-2-oleoylphosphatidylcholine and dioleoylphosphatidylglycerol (molar ratio 9/1) as described in Example 9 - Formulation Code B. In Table 7, the preferred formulations are A, B & C. B and C are particularly preferred formulations. As for non-extractable hCT formulations E and F are preferred. Empty liposomes were prepared similarly using buffer which lacked hCT. Rats were injected subcutaneously with the liposome/hCT preparation alone at a dose of 1.44 U/kg hCT and 1.4 umol lipid/kg. Alternatively, rats received the 0.69 U/kg of liposomal hCT and 144 umol of empty liposome lipid/kg. Blood samples were obtained at various times and the serum was frozen and thawed 5 times and hCT was measured by radioimmunoassay using an assay kit obtained from the Nichols Institute,

San Juan Capistrano, CA. The results are shown in FIG. 1.

With liposome hCT alone, peak levels of hCT were noted at 8 hrs., 159 pg/ml. At 24 and 48 hrs. hCT levels were noted of 19.6 and 24.1 pg/ml, representing a significant increase over the basal level, 12.8 pg/ml.

When empty liposome were injected together with the liposomal hCT, much higher levels of hormone were measured even though the dose of liposomal hCT was lower (0.69 versus 1.44 U/kg). At 8 hrs. the peak level of hCT was 1698 pg/ml, more than 10 times greater than with liposomal hCT alone. At 24 and 48 hours, hCT levels of 466 and 31 pg/ml were found versus a basal level of 9.0 pg/ml. As is apparent, coadministration of empty liposomes together with liposomal hCT greatly increased serum levels of hCT.

<u>Example 12</u>

In this example, graded doses of empty liposomes of the type used in Example 11 were coadministered with 1.44 U/kg of liposomal hCT as prepared in the same example and serum hCT levels assayed at 6, 18, 24 and 48 hours. As is shown in FIG. 2, it is apparent that there is a graded increase in the amount of hCT found in the serum which varies directly with the amount of lipid given. For example, at 6 hours with 4.7 umol of lipid/kg 329 pg/ml of hCT was detected. When the dose of coadministered lipid increased to 14.2, 47.3 and 142 umol/kg, serum hCT levels of 678, 2,290 and 5,380 were found as shown in FIG. 2. This example shows that graded doses of coadministered liposomal lipid increase the serum level of hCT at various sampling times.

Empty liposomes were also found to be effective in increasing the shelf life of therapeutic peptides or proteins which are dispersed in solution in a container. The stabilization provided by the empty liposomes is believed to be due to the liposomes preventing adsorption of the peptide or protein on the container surface, furthermore preventing the peptide from aggregation or denaturation. This aspect of the present invention is applicable to wide variety of therapeutic peptides and proteins, including, but not limited to, the peptides and proteins previously listed. The use of liposomes to protect recombinant peptides and proteins is especially useful.

The use of liposomes as a diluent is especially well-suited for increasing the shelf-life of solutions where the concentration of peptide or protein is between about1 nanogram per ml to 10 milligrams per ml.

A wide variety of lipids may be used to form the liposomes which are added as a diluent. Suitable lipids include, but are not limited to, the lipids previously listed as being acceptable for making liposomes in accordance with the present invention. The limitations previously described with respect to net charge, size and leakage rates in serum or lymph fluid do not apply. Accordingly, many other lipids can be used provided that they can be formed into liposomes.

In the liposomes of the present invention phosphatidylcholine is preferred. Synthetic phospholipid can be used as well as those purified from natural sources. The fatty acid groups of the synthetic phospholipid are preferably mono-unsaturated, and have a chain length of either 16 or 18. Alternatively, phospholipid having greater degrees of unsaturation, for example, having from 1 to 6 double bonds and those with chain lengths of from 12 to 24 carbons may be used. Proportions of lipids are expressed as mole percent of total lipids.

Negatively charged phospholipid may be added to the lipid phase in concentrations up to about 50 mole percent. In a preferred embodiment, a negatively charged species is phosphatidylglycerol, which is added to the lipid phase in the amount of at least about 10 mole percent, and preferably at least about 10 mole percent of the total lipids, with the remainder comprised of phosphatidyl choline. Up to 50 mole percent of cholesterol may also be included.

The primary liposomes are sized to homogeneity, or at least to a narrow distribution of diameters within the biologically suitable range, by a freeze-thaw-extrusion process essentially according to Mayer, L. et al., Biochim. Biophys. Acta 858:161-168 (1986), as described in Example 1. The liposomes may be frozen by chilling to a temperature at which the fatty acid chains are no longer fluid. Suitable freezing conditions may be provided by a bath of dry ice in acetone, an alcohol, or other suitable solvent which will provide a fluid system at the melting point of dry ice, about -70°C. Under these conditions the aqueous diluent buffer also freezes. Thawing of the preparations may be conveniently carried out at room temperature. Alternatively, the preparation may be frozen by immersion in liquid nitrogen and thawing may be carried out in a water bath to provide a temperature above room temperature at, for example, 37°C.

Suitable filters for the extrusion may be of any type prepared for microfiltration, such as those manufactured by Nucleopore, Inc. (Pleasanton, California), having a uniform pore size and manufactured of a non-contaminating material. Filters used for the procedure described in Exaple 1 were typically of pore sizes either 50 nm or 200 nm.

Filters having larger or smaller pore sizes may be used to size preparations of liposomes to larger or

smaller mean diameters. Preferably the filters have a uniform pore size in the range of form about 30 to 400 nm. Most preferably, the filter has a pore size by extrusion through a ceramic filter such as, for example, one of the types described in U.S. Patent No. 4,737,323 to Martin. The extrusion is carried out under pressure to facilitate the flow of the preparation. Suitable pressures may range from 100 to 700 psi. The pressure applied is greater or lesser as required to extrude liposomes through filters of different pore sizes.

The amount of liposomes added to the peptide or protein solution may be varied depending upon the particular liposome used and the concentration of peptide or protein. Typically, the amount of liposome will be between about 10 micrograms to 200 milligrams per ml. The concentration of liposome necessary to provide an optimum increase in shelf life can be easily determined experimentally.

FIGS. 3-16 depict the results of the following examples where various peptide and protein solutions were treated with liposomes to increase shelf life. The liposomes used in all of the examples were the same empty liposomes described in Examples 8 and 9.

Example 13

Calcitonin was diluted from a 10 mg/ml aqueous stock solution to 40 ug/ml with 0.1M phosphate buffer, pH 7.4. This diluted stock solution was serially diluted down to a theoretical concentration of 0.625 ug/ml and was assayed. Glass test tubes were used as the receiving containers. Each serial dilution was assayed using a fluorescamine assay. An aliquot of the 40 ug/ml dilution was retained and was similarly serially diluted and assayed on the second day. A considerable reduction in the calcitonin content of the samples assayed on the second day was noted for each dilution. As shown in FIG. 3, significant curvature of

the day 1 standard curve indicates loss of peptide already on this day. The second day curve shows even greater losses.

Example 14

The same procedure as in Example 13 was followed, except that the containers were polypropylene tubes. The results shown in FIG. 4 again show a continued marked decrease in calcitonin after both 1 and 2 days.

Example 15

Examples 13 and 14 were repeated except that the empty liposomes of Examples 11 and 12 were added to provide solutions which a final concentration of 7.5 mM liposomes. As shown in FIGS. 5 and 6, the problem with decreasing calcitonin concentration on the second day was virtually eliminated.

Examples 16-22

These examples were all conducted in the same manner as Example 15 except that different peptides were substituted for calcitonin. As shown in FIGS. 7A and B, ACTH losses after two days in glass or polypropylene containers were at a lower level when 2.5 mM liposomes were added. FIGS. 8A and B show the same reduced losses for liposome atriopeptin after two days. FIGS. 9A and B show the results for parathyroid hormone (PTH), while FIGS. 10A and B, FIGS. 11A and B, FIGS. 12A and B and FIGS. 13A and B show the reduced losses for somatostatin, enkephalin, oxytocin and vasopressin, respectively. The results all show low levels of protein or peptide losses after two days in those solutions containing liposomes as a diluent. 100% control was determined by using 2.5 Zwittergent 3-14 (w/v)(Calbiochem).

Example 23

A number of different peptides having amino acid numbers ranging from 5 to 34 were substituted for the calcitonin in Example 15. As shown in FIG. 14, the losses after two days in polypropylene containers for

the various peptides treated with liposomes was much less than for the phosphate solutions without liposomes.

Having thus described exemplary embodiments of the present invention, it should be noted by those skilled in the art that the within disclosures are exemplary only and that various other alternatives, adaptations and modifications may be made within the scope of the present invention. Accordingly, the present invention is not limited to the specific embodiments as illustrated herein. Furthermore, the present invention may be reasonably expected to provide extended delivery of therapeutic peptides and proteins either natural, synthetic or of a hybrid nature which will be discovered in the future. Therefore, regardless of whether a therapeutic peptide or protein is presently known, or whether it becomes known in the future, the methods of formulating liposomes for extended delivery according to this disclosure will be apparent to those of skill in the art and their incorporation into liposomes is broadly enabled by this invention.

WHAT IS CLAIMED IS:

1. A composition for prolonging the bioavailability of therapeutic peptides or proteins, said composition comprising a therapeutic peptide or protein encapsulated in a unilamellar or oligolamellar liposome vesicle wherein said liposome vesicle stable in lymph fluid, but unstable in serum or plasma and wherein the net electric charge of said liposome vesicle is negative or neutral and said liposome vesicle contains less than 10 mole percent cholesterol.

2. A composition according to claim 1 wherein said liposome vesicle includes at least one percent of a lipid selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid, phosphatidylglycerol, phosphatidylserine, phosphatidylinositol, sphingomyelin, and dimyristoyl-, dipalmitoyl-, distearoyl-, dioleoyl-, and 1-palmitoyl, 2-oleoyl- analogues of phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidic acid and phosphatidylserine.

3. A composition according to claim 1 wherein the outside diameter of said lipid vesicles is from between about 20 to 200 nanometers.

4. A composition according to claim 1 wherein the molecular weight of said therapeutic peptide or protein is between about 500 to 100,000.

5. A composition according to claim 2 wherein said vesicle comprises phosphatidylcholine and phosphatidylglycerol.

6. A composition according to claim 2 wherein said vesicle comprises dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol.

7. A composition for administration to a mammal comprising:

a therapeutic peptide or protein encapsulated in a unilamellar or oligolamellar liposome vesicle wherein said liposome is stable in lymph fluid, but unstable in serum or plasma and wherein the net electric charge of said liposome vesicle is negative or neutral and said liposome vesicle contains less than 10 mole percent cholesterol; and

a pharmaceutically acceptable isotonic carrier solution for said liposome vesicles wherein the osmolarity of said carrier solution is between about 200 to 400 mosmole.

8. A composition according to claim 7 wherein said liposome vesicle includes at least one percent of a lipid selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid, phosphatidylglycerol, phosphatidylserine, phosphatidylinositol, sphingomyelin, and dimyristoyl-, dipalmitoyl-, distearoyl-, dioleoyl-, and 1-palmitoyl, 2-oleoyl- analogues of phosphatidylcholine, phosphatidylethanolamine, phosphatidyllglycerol, phosphatidic acid and phosphatidylserine.

9. A composition according to claim 7 wherein the outside diameter of said lipid vesicles is from between about 20 to 200 nanometers.

10. A composition according to claim 7 wherein the molecular weight of said therapeutic peptide or protein is between about 500 to 100,000.

11. A composition according to claim 8 wherein said vesicle comprises phosphatidylcholine and phosphatidylglycerol.

12. A composition according to claim 8 wherein said vesicle comprises dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol.

13. A method for prolonging the bioavailability of a therapeutic peptide or protein comprising the step of encapsulating said peptide or protein in a unilamellar or oligolamellar liposome vesicle to form an encapsulated peptide or protein wherein said liposome vesicle is stable in lymph fluid, but unstable in serum or plasma and wherein the net electric charge of said liposome vesicle is negative or neutral and said liposome vesicle contains less than 10 mole percent cholesterol.

14. A method according to claim 13 including the additional step of dispersing said encapsulated peptide or protein in a pharmaceutically acceptable isotonic carrier solution to form an injectable medicament wherein the osmolarity of said carrier solution is between about 200 to 400 mosmol.

15. A method according to claim 14 including the additional step of subcutaneously injecting said medicament into said mammal.

16. A method according to claim 13 wherein said liposome vesicle includes at least one percent of a

lipid selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid, phosphatidylglycerol, phosphatidylserine, phosphatidylinositol, sphingomyelin, and dimyristoyl-, dipalmitoyl-, distearoyl-, dioleoyl-, and 1-palmitoyl, 2-oleoyl- analogues of phosphatidylcholine, phosphatidylethanolamine, phosphatidyllglycerol, phosphatidic acid and phosphatidylserine.

17. A method according to claim 13 wherein the outside diameter of said lipid vesicles is from between about 20 to 200 nanometers.

18. A method according to claim 13 wherein the molecular weight of said therapeutic peptide or protein is between about 500 to 100,000.

19. A method according to claim 16 wherein said vesicle comprises phosphatidylcholine and phosphatidylglycerol.

20. A method according to claim 16 wherein said vesicle comprises dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol.

21. A composition of matter comprising a therapeutic agent encapsulated in a liposome wherein the improvement comprises increasing the bioavailability of said therapeutic agent by adding empty liposomes to said composition.

22. An improved composition according to claim 21 wherein the weight ratio of encapsulated liposomes to empty liposomes is between about 1:1 and 1:10,000.

23. An improved composition according to claim 21 wherein said therapeutic agent is a therapeutic peptide or protein having a molecular weight of between about 500 to 100,000.

24. An improved composition according to claim 21 wherein said empty liposome includes lipid selected from the group consisting of phosphatidylcholine, phosphatidlethanolamine, phosphatidic acid, phosphatidylglycerol, phosphatidylserine, phosphatidylinositol, sphingomyelin, and dimyristoyl-, dipalmitoyl-, distearoyl-dioleoyl-, and 1-palmitoyl, 2-oleoyl- analogues of phosphatidylcholine, phosphatidylethanolamine, phosphatidyllglycerol, phosphatidic acid and phosphatidylserine.

25. An improved composition according to claim 21 whrein said therapeutic agent is calcitonin.

26. A method for administering liposome encapsulated therapeutic agents to a mammal, wherein the improvement comprises adding empty liposomes to said liposome encapsulated therapeutic agent prior to administration to said mammal to provide increased bioavailability of said therapeutic agent.

27. An improved method according to claim 26 wherein the weight ratio of encapsulated liposomes to empty liposomes is between about 1:1 and 1:10,000.

28. An improved method according to claim 26 wherein said therapeutic agent is a therapeutic peptide or protein having a molecular weight of between about 500 to 100,000.

29. An improved method according to claim 26 wherein said empty liposome includes a lipid selected

from the group consisting of phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid, phosphatidylglycerol, phosphatidylserine, phosphatidylinositol, sphingomyelin, and dimyristoyl-, dipalmitoyl-, distearoyl-, dioleoyl-, and 1-palmitoyl, 2-oleoyl-analogs of phosphatidylcholine, phosphatidylethanolamine, phosphatidyllglycerol, phosphatidic acid and phosphatidylserine.

30. A composition of matter comprising a therapeutic peptide or protein suspended in a solution contained in a vessel wherein the improvement comprises extending the shelf life of said therapeutic peptide or protein by adding a sufficient amount of liposomes to said solution to extend the shelf life of said therapeutic peptide or protein.

31. An improved composition according to claim 30 wherein the concentration of said therapeutic peptide or protein in said solution is between about 1 nanogram per ml and 10 milligrams per ml.

32. An improved composition according to claim 29 wherein said liposome includes a lipid selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid, phosphatidylglycerol, phosphatidylserine, phosphatidylinositol, sphingomyelin, and dimyristoyl-, dipalmitoyl-, distearoyl-, dioleoyl-, and 1-palmitoyl, 2-oleoyl- analogues of phosphatidylcholine, phosphatidylethanolamine, phosphatidyllglycerol, phosphatidic acid and phosphatidylserine.

33. An improved composition according to claim 30 wherein the concentration of said liposome in said solution is between about 10 micrograms per ml to 200 milligrams per ml.

34. An improved composition according to claim 31 wherein the concentration of said liposome in said solution is between about 10 micrograms per ml to 200 milligrams per ml.

35. An improved composition according to claim 30 wherein said therapeutic protein is calcitonin.

36. An improved composition according to claim 35 wherein said liposome includes 1-palmitoyl-2-oleoyl-phosphatidylcholine and dioleoylphosphatidylglycerol.

37. A method for increasing the shelf life of therapeutic peptides or proteins dispersed in a solution in a container, said method comprising the step of adding a sufficient amount of liposomes to said solution to extend the shelf life of said therapeutic peptides or proteins.

38. A method according to claim 37 wherein the concentration of said therapeutic peptide or protein is between about 1 nanogram per ml and 10 milligrams.

39. A method according to claim 37 wherein said liposome includes a lipid selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid, phosphatidylglycerol, phosphatidylserine, phosphatidylinositol, sphingomyelin, and dimyristoyl-, dipalmitoyl-, distearoyl-, dioleoyl-, and 1-palmitoyl, 2-oleoyl- analogues of phosphatidylcholine, phosphatidylethanolamine, phosphatidyllglycerol, phosphatidic acid and phosphatidylserine.

40. A method according to claim 37 wherein the amount of liposome added to said solution is between about 10 micrograms per ml and 200 milligrams per ml.

41. A method according to claim 38 wherein the amount of liposome added to said solution is between about 10 micrograms and 200 milligrams per ml.

42. A method according to claim 37 wherein said therapeutic protein is calcitonin.

43. A method according to claim 42 wherein said liposome includes 1-palmitoyl-2-oleoylphosphatidylglycerol and dioleoylphosphatidylcholine.

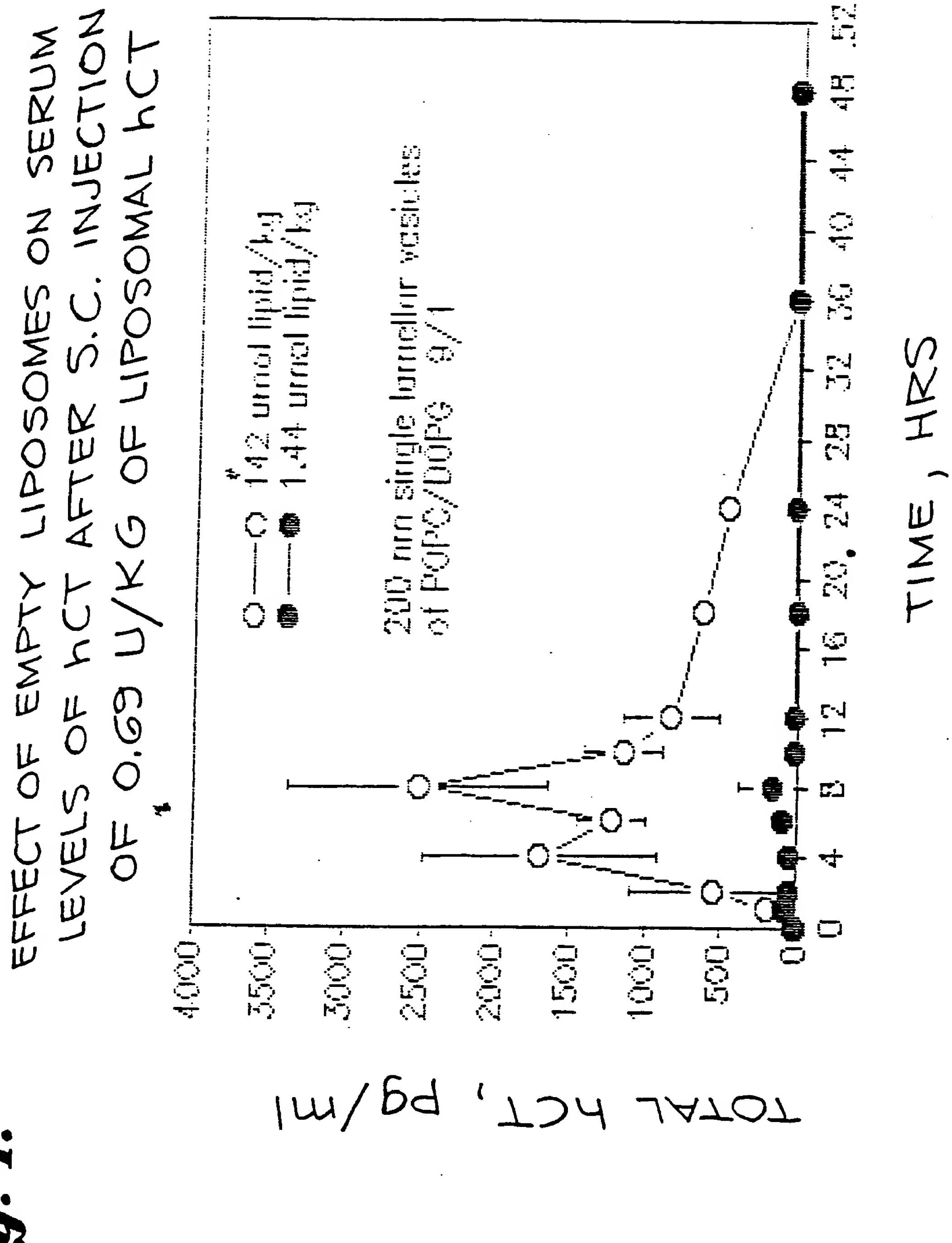

EFFECT OF EMPTY LIPOSOMES ON SERUM LEVELS OF hCT AFTER S.C. INJECTION OF 0.69 U/KG OF LIPOSOMAL hCT
142 umol lipid/kg
1.44 umol lipid/kg
200 nm single lamellar vesicles of POPC/DOPG 9/1
TOTAL hCT, pg/ml
4000
3500
3000
2500
2000
1500
1000
500
0
0
4
8
12
16
20
24
28
32
36
40
44
48
52
TIME, HRS

Fig. 1.

EFFECT OF EMPTY LIPOSOMES ON SERUM LEVELS OF hCT AFTER SC INJECTION OF 1.44 U/KG OF LIPOSOMAL hCT

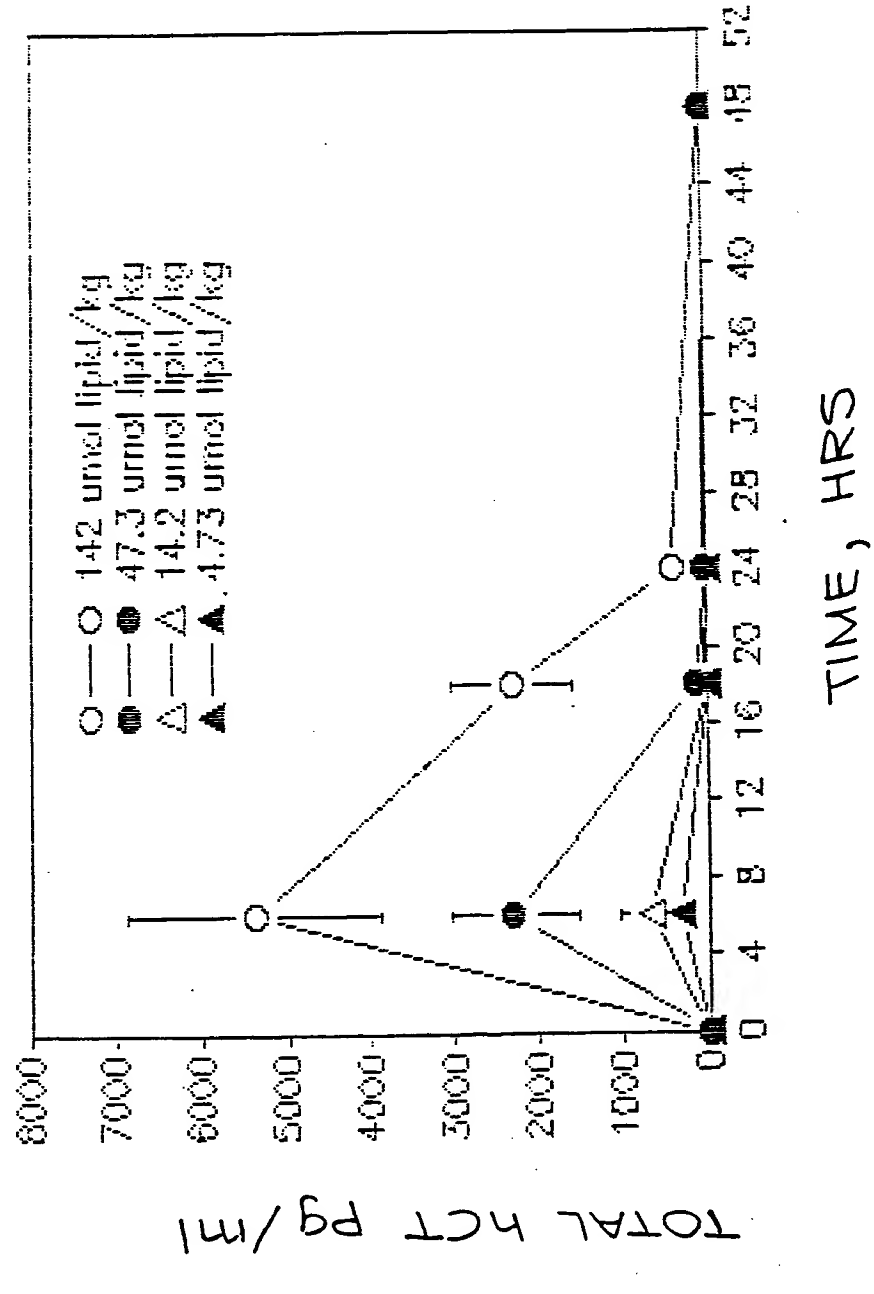
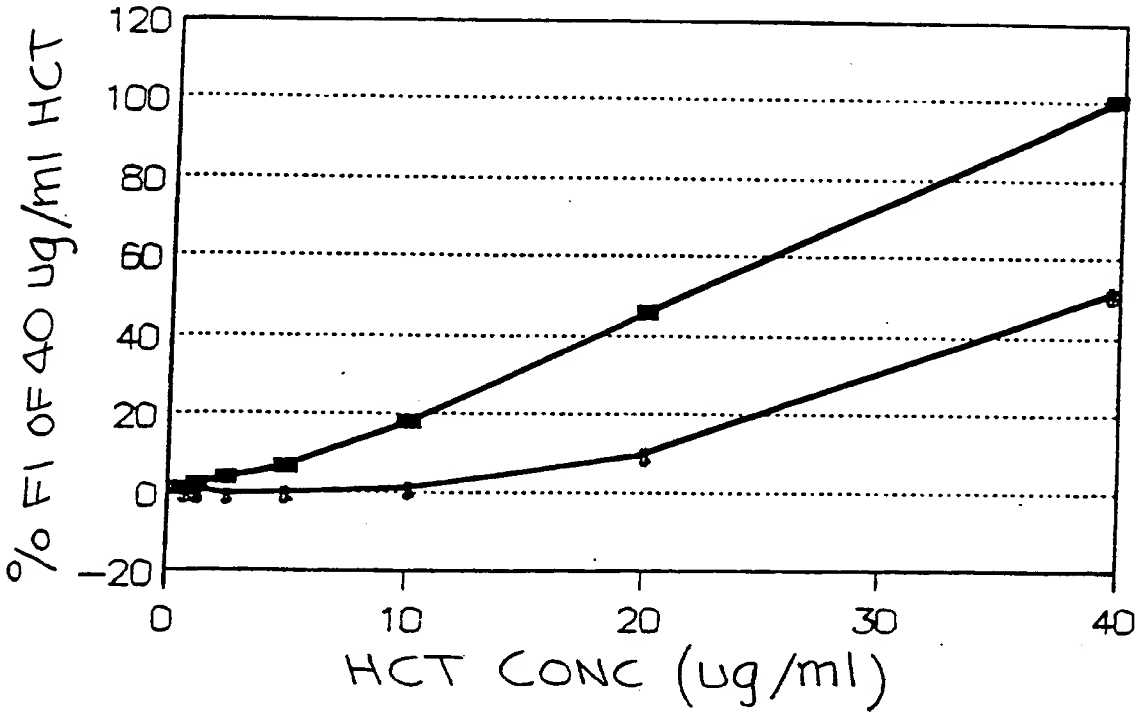


Fig. 2

Fig. 3.

SERIAL DIL OF HCT IN 0.1 M PHOSPHATE
ADSORPTION LOSS IN GLASS TUBE



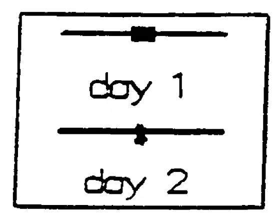


Fig. 4.

SERIAL DIL OF HCT IN 0.1 M PHOSPHATE
ADSORPTION LOSS IN POLYPROPYLENE TUBE

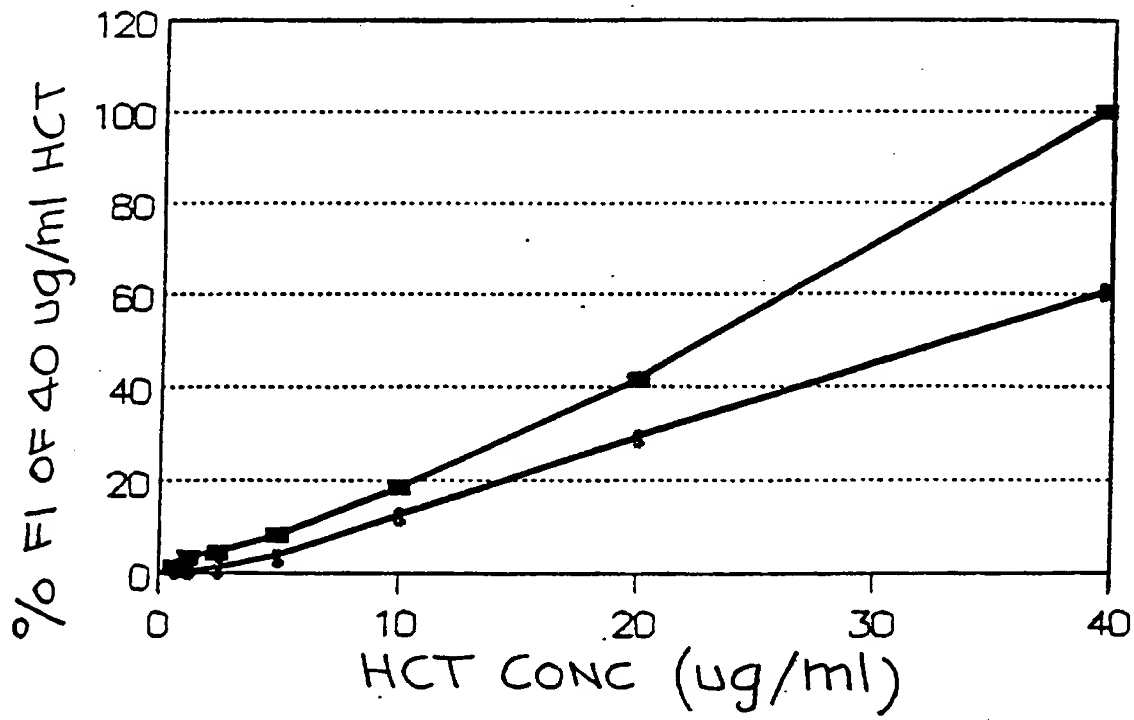


Fig. 5. SERIAL DIL OF HCT IN EMPTY LIPOSOME ADSORPTION LOSS IN GLASS TUBE

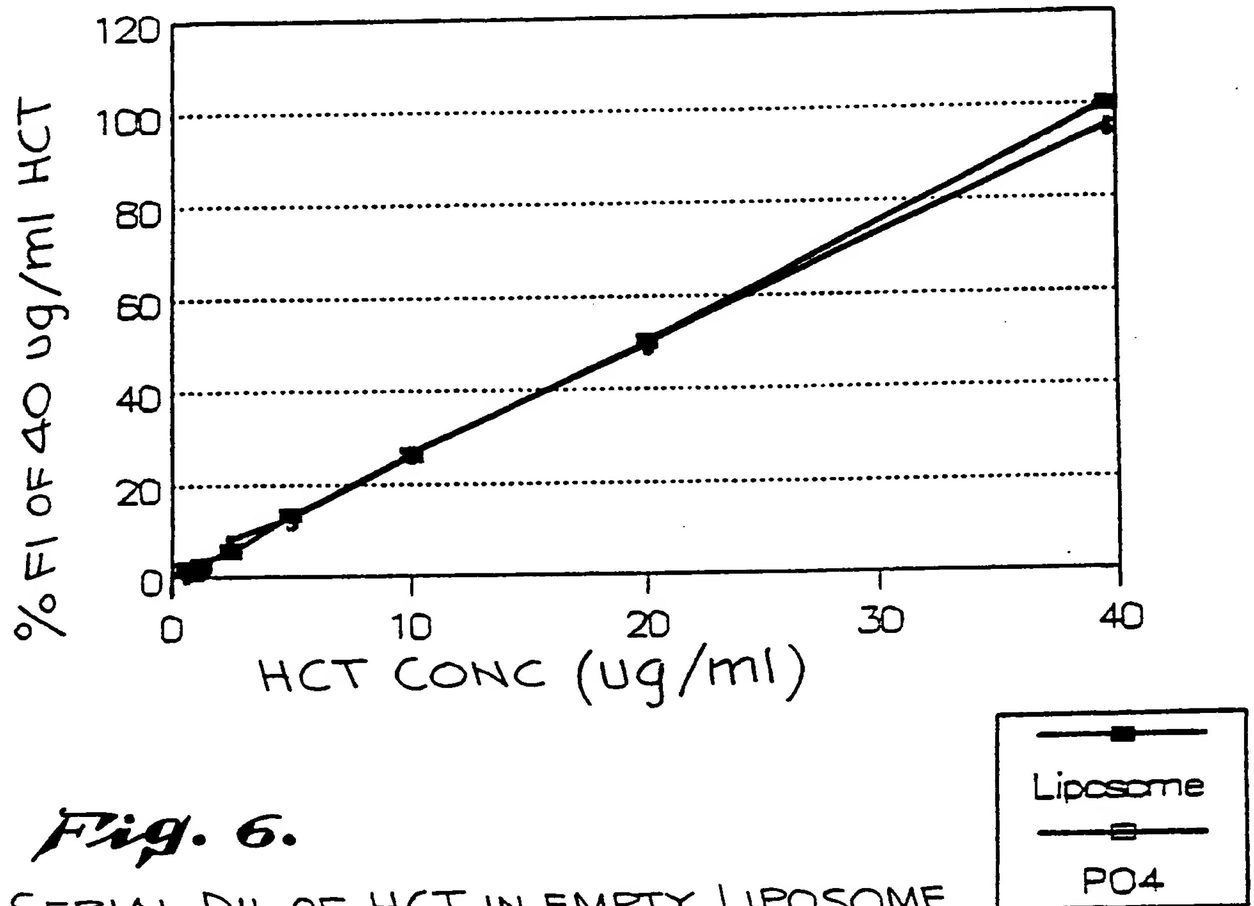


Fig. 6.

SERIAL DIL OF HCT IN EMPTY LIPOSOME ADSORPTION LOSS IN POLYPROPYLENE TUBE

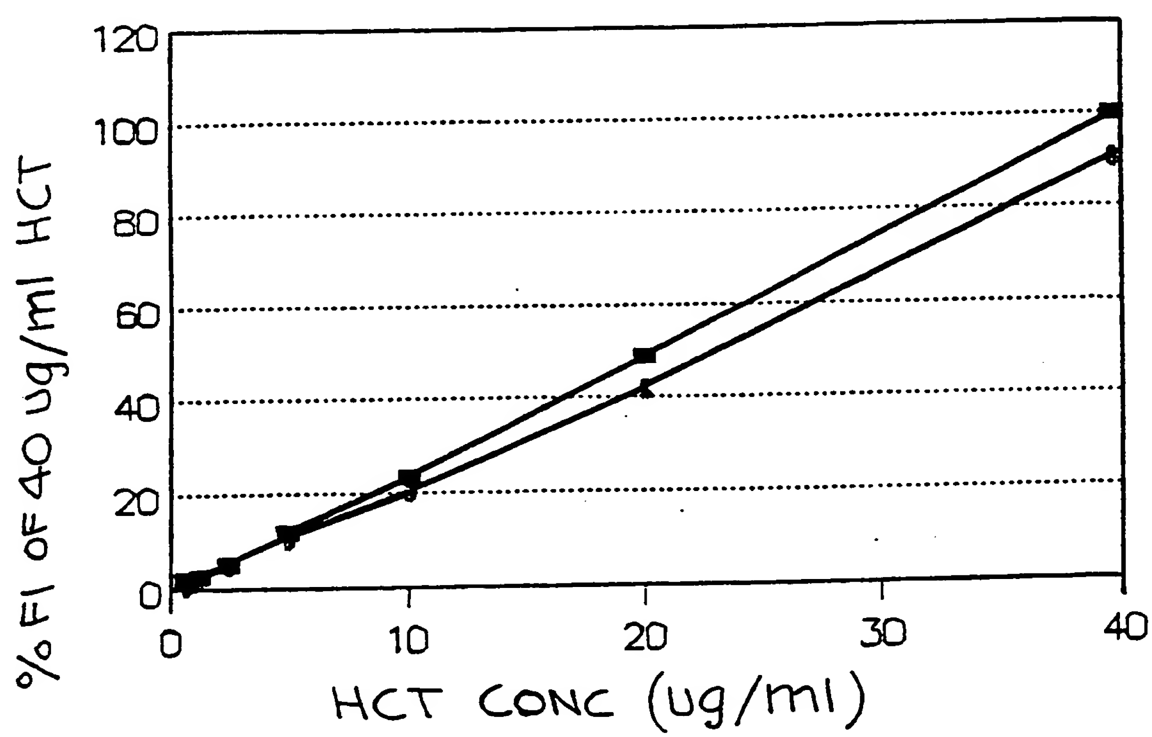
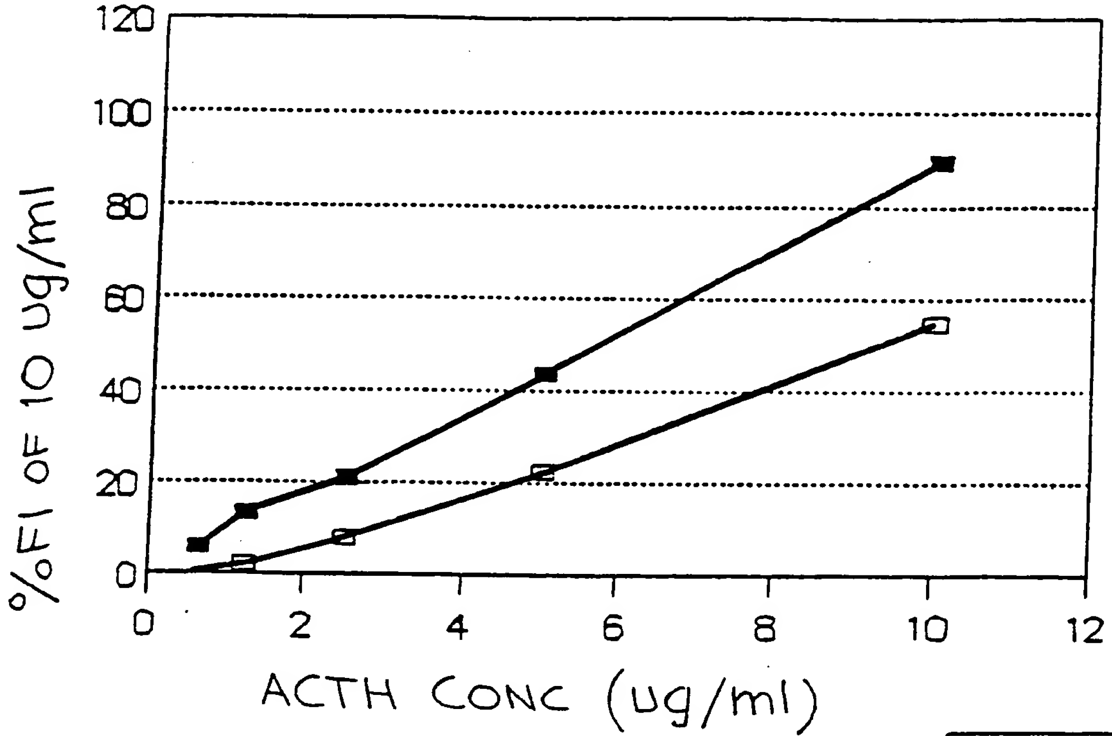


Fig. 7.a

ACTH SERIAL DIL
ADSORPTION LOSSES ONTO PP TUBE



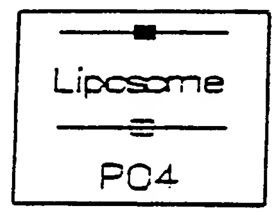


Fig. 7.b

ACTH SERIAL DIL
ADSORPTION LOSSES ONTO GLASS TUBE

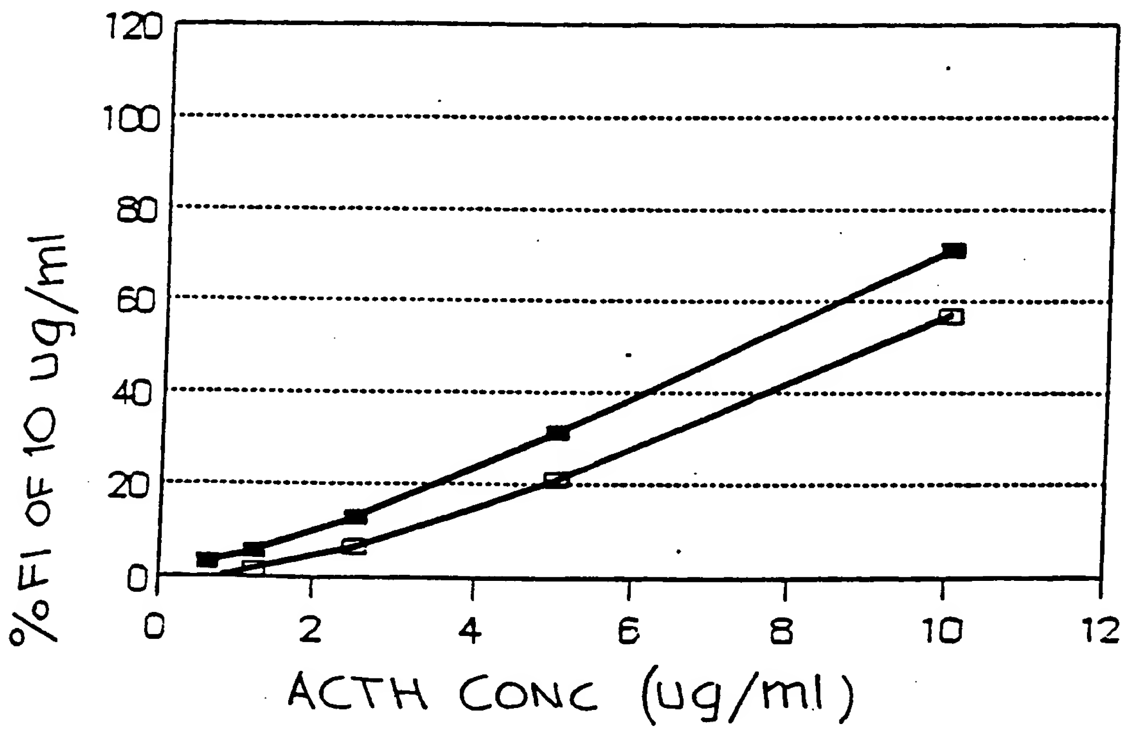
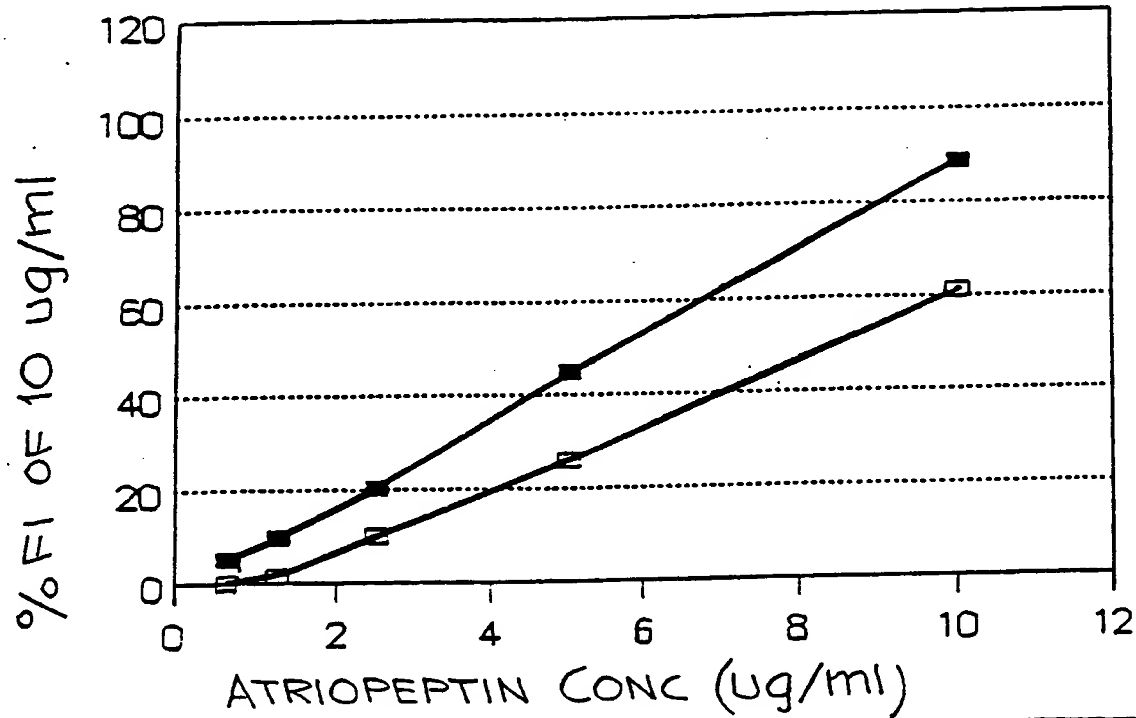


Fig. 8a

ATRIOPEPTIN SERIAL DIL
ADSORPTION LOSSES ONTO PP TUBE



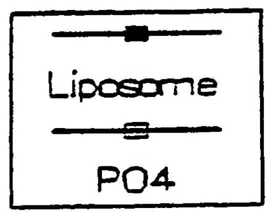


Fig. 8b

ATRIOPEPTIN SERIAL DIL
ADSORPTION LOSSES ONTO GLASS TUBE

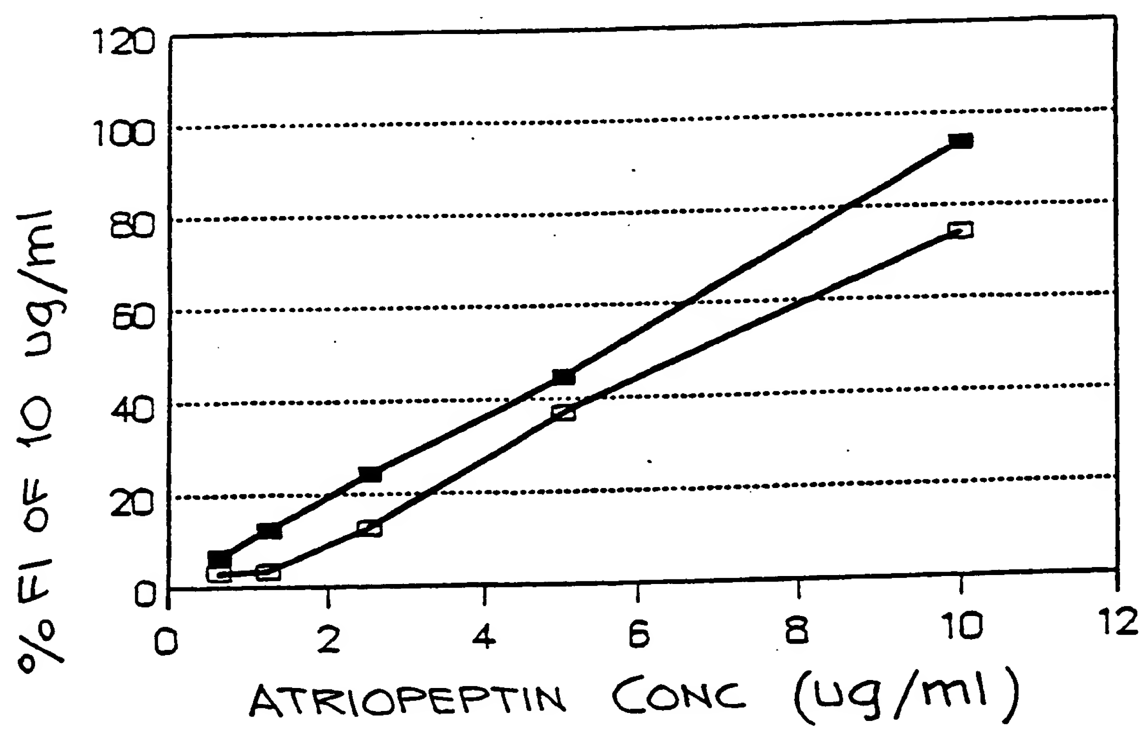


Fig. 9a

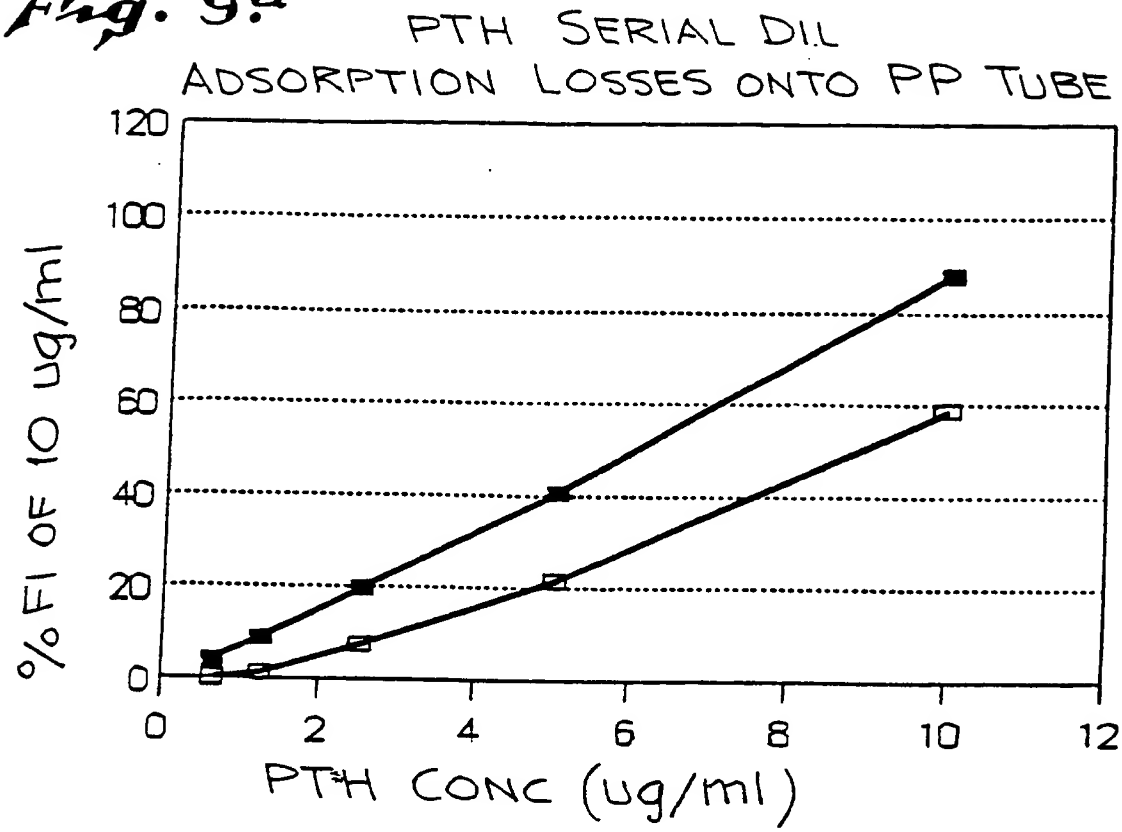

PTH SERIAL DIL
ADSORPTION LOSSES ONTO PP TUBE
120
100
80
60
40
20
0
% FI OF 10 ug/ml
0
2
4
6
8
10
12
PTH CONC (ug/ml)

Fig. 9b

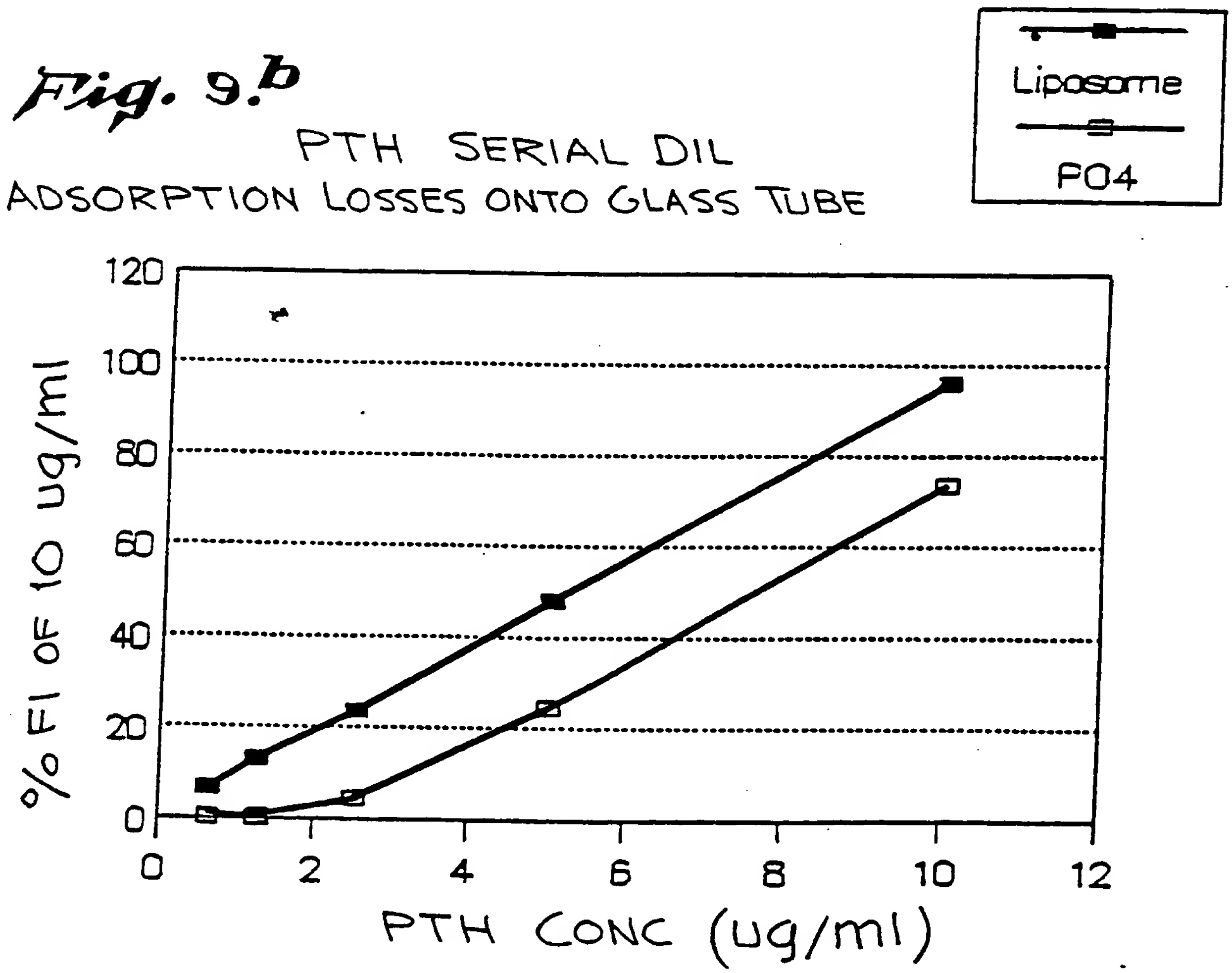
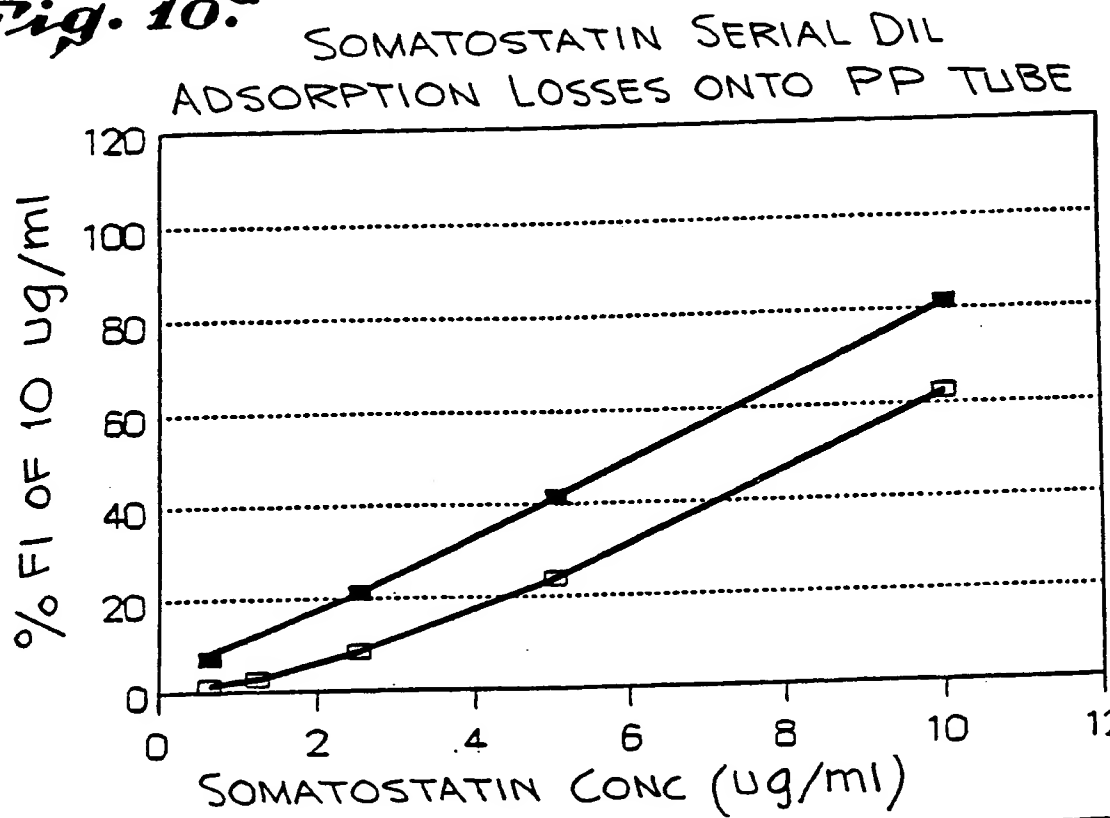

PTH SERIAL DIL
ADSORPTION LOSSES ONTO GLASS TUBE
Liposome
PO4
120
100
80
60
40
20
0
% FI OF 10 ug/ml
0
2
4
6
8
10
12
PTH CONC (ug/ml)

Fig. 10a



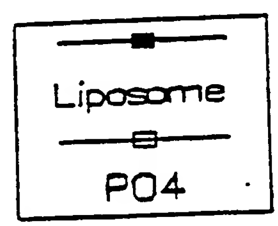


Fig. 10b

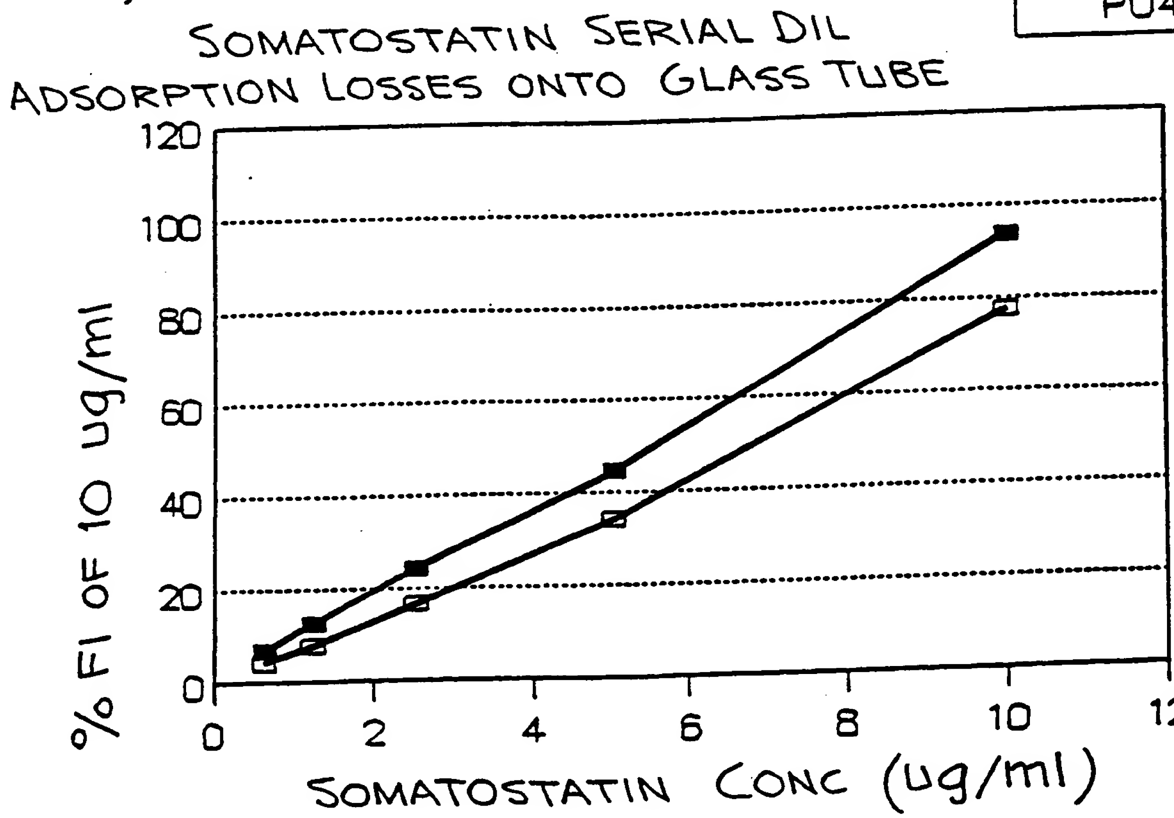
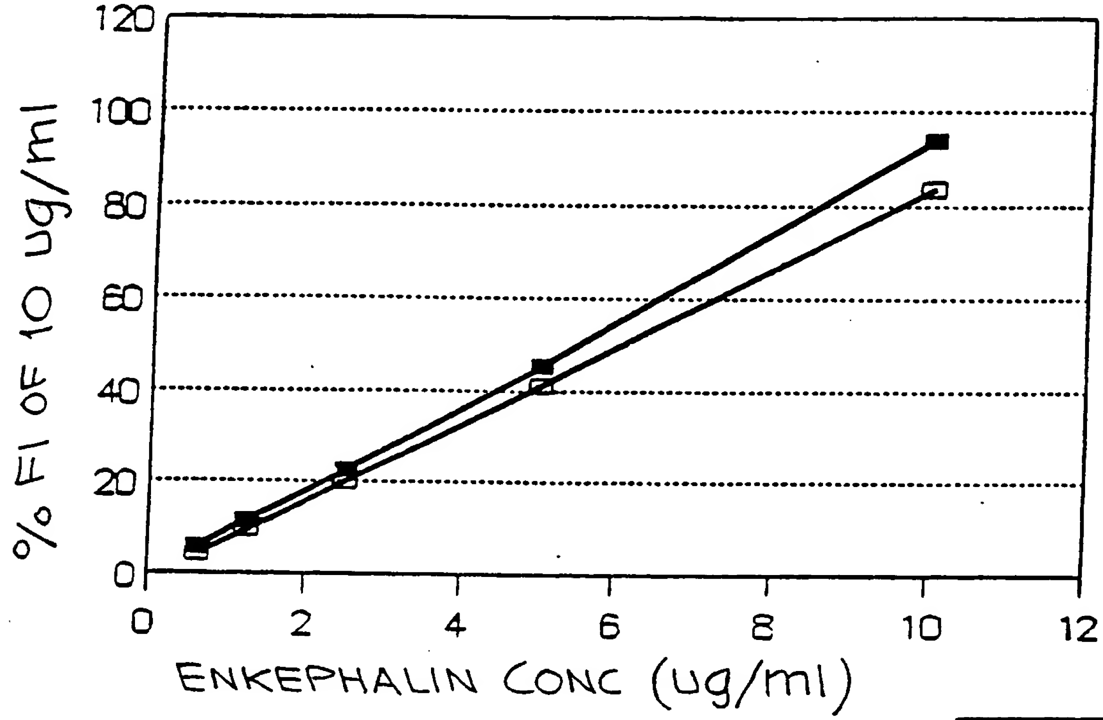


Fig. 11a

ENKEPHALIN SERIAL DIL
ADSORPTION LOSSES ONTO PP TUBE



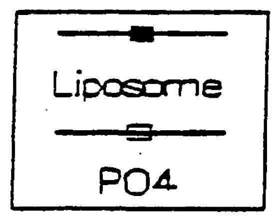


Fig. 11b

ENKEPHALIN SERIAL DIL
ADSORPTION LOSSES ONTO GLASS TUBE

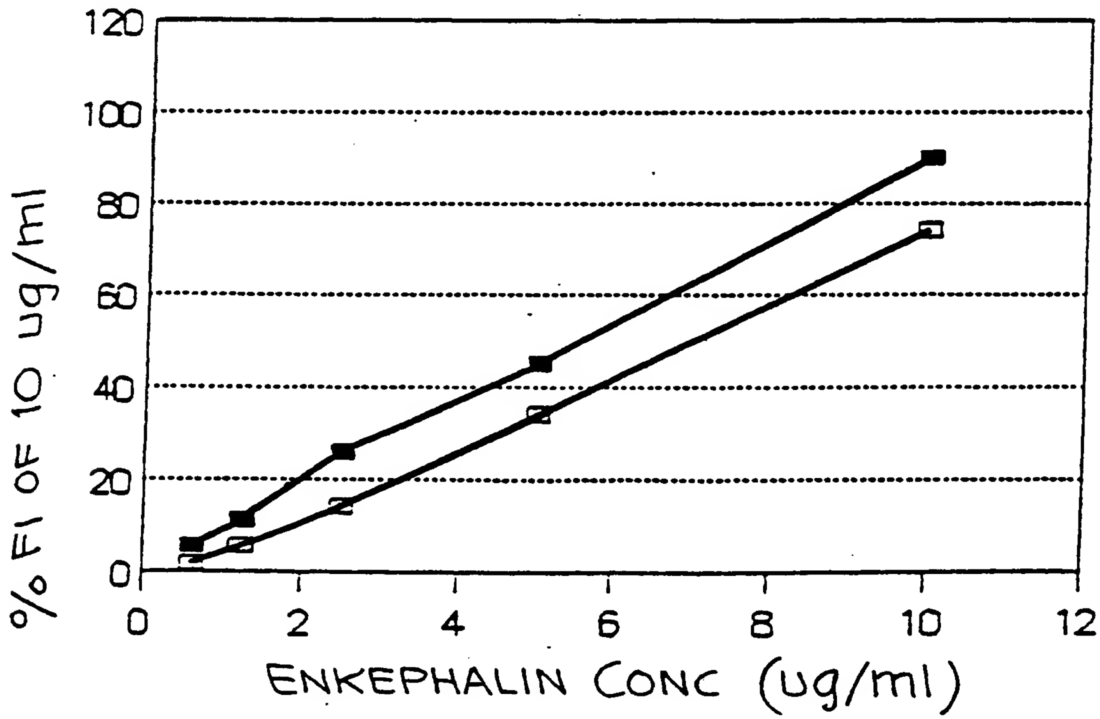
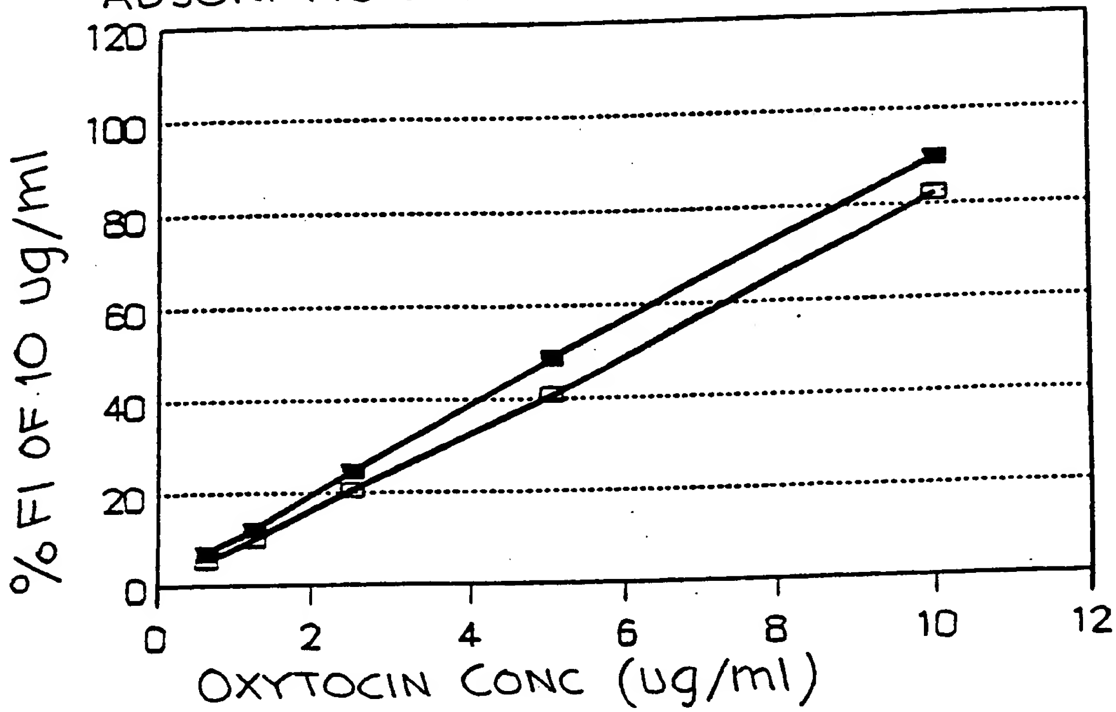


Fig. 12.a

OXYTOCIN SERIAL DIL
ADSORPTION LOSSES ONTO PP TUBE



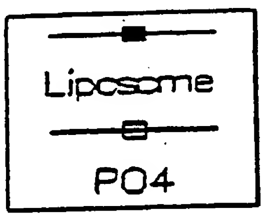


Fig. 12.b

OXYTOCIN SERIAL DIL
ADSORPTION LOSSES ONTO GLASS TUBE

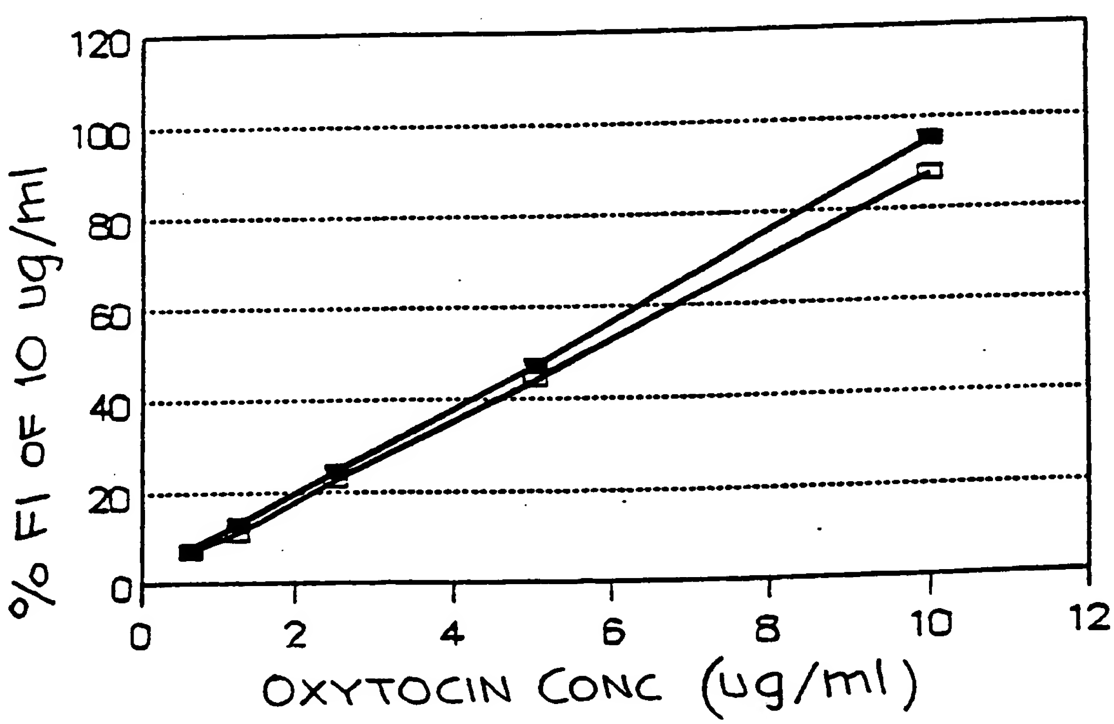


Fig. 13.a

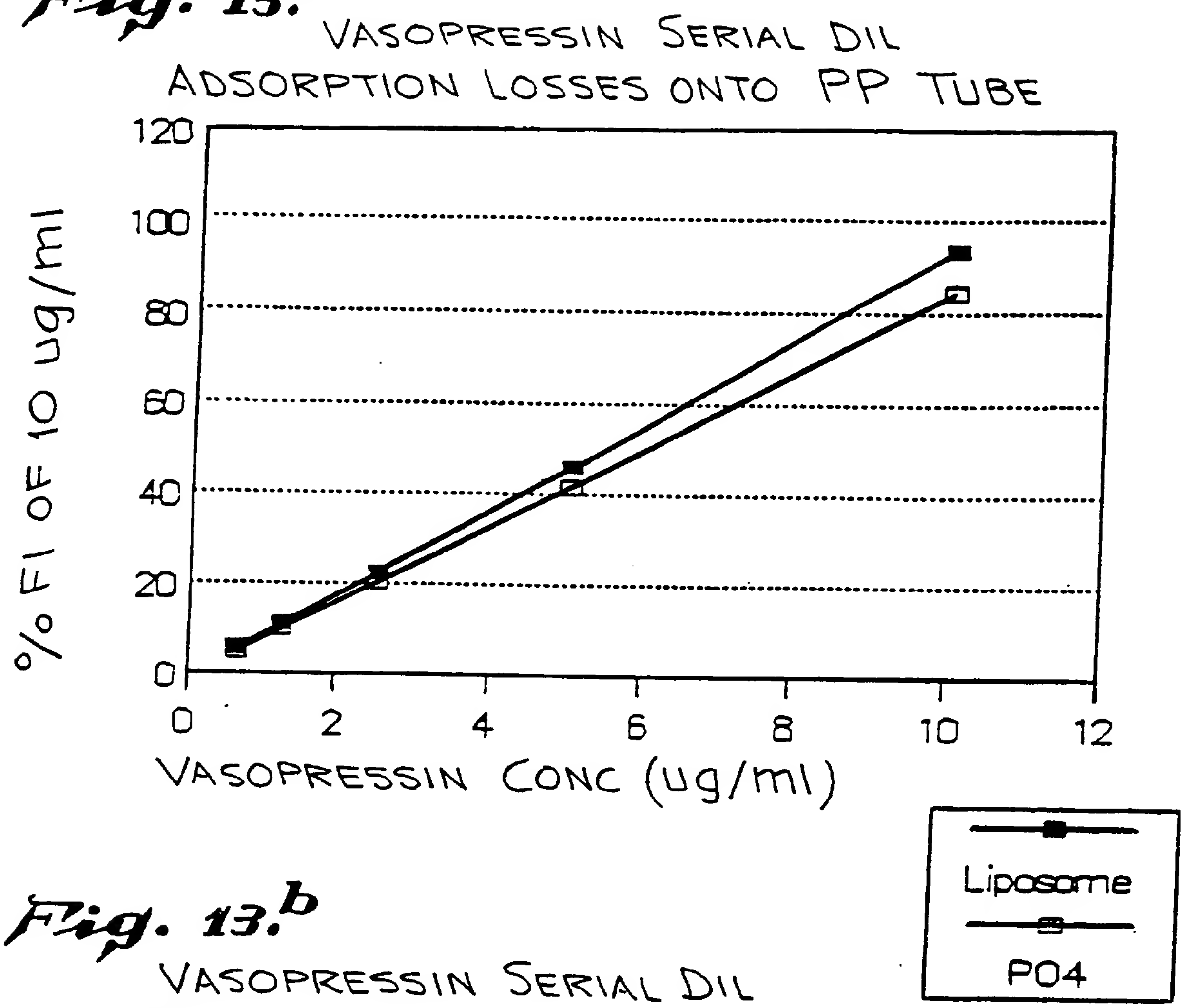


Fig. 13.b

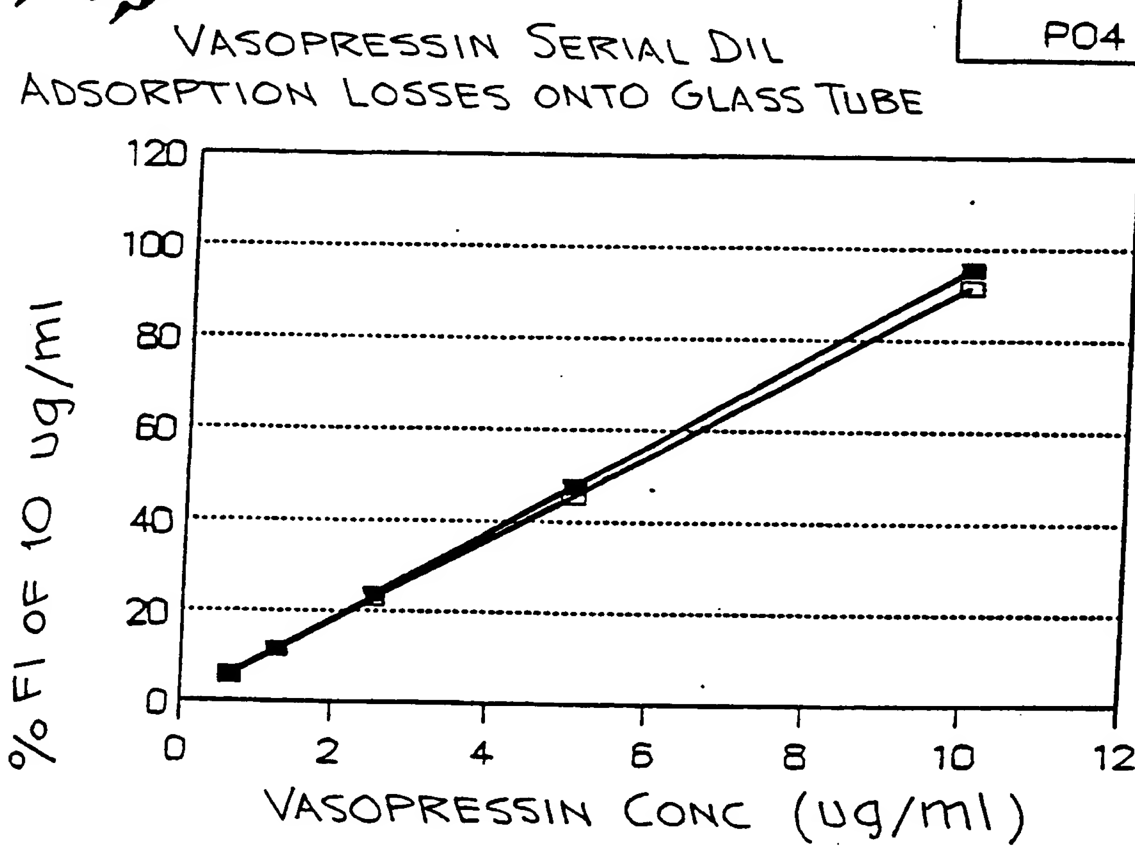


Fig. 14.

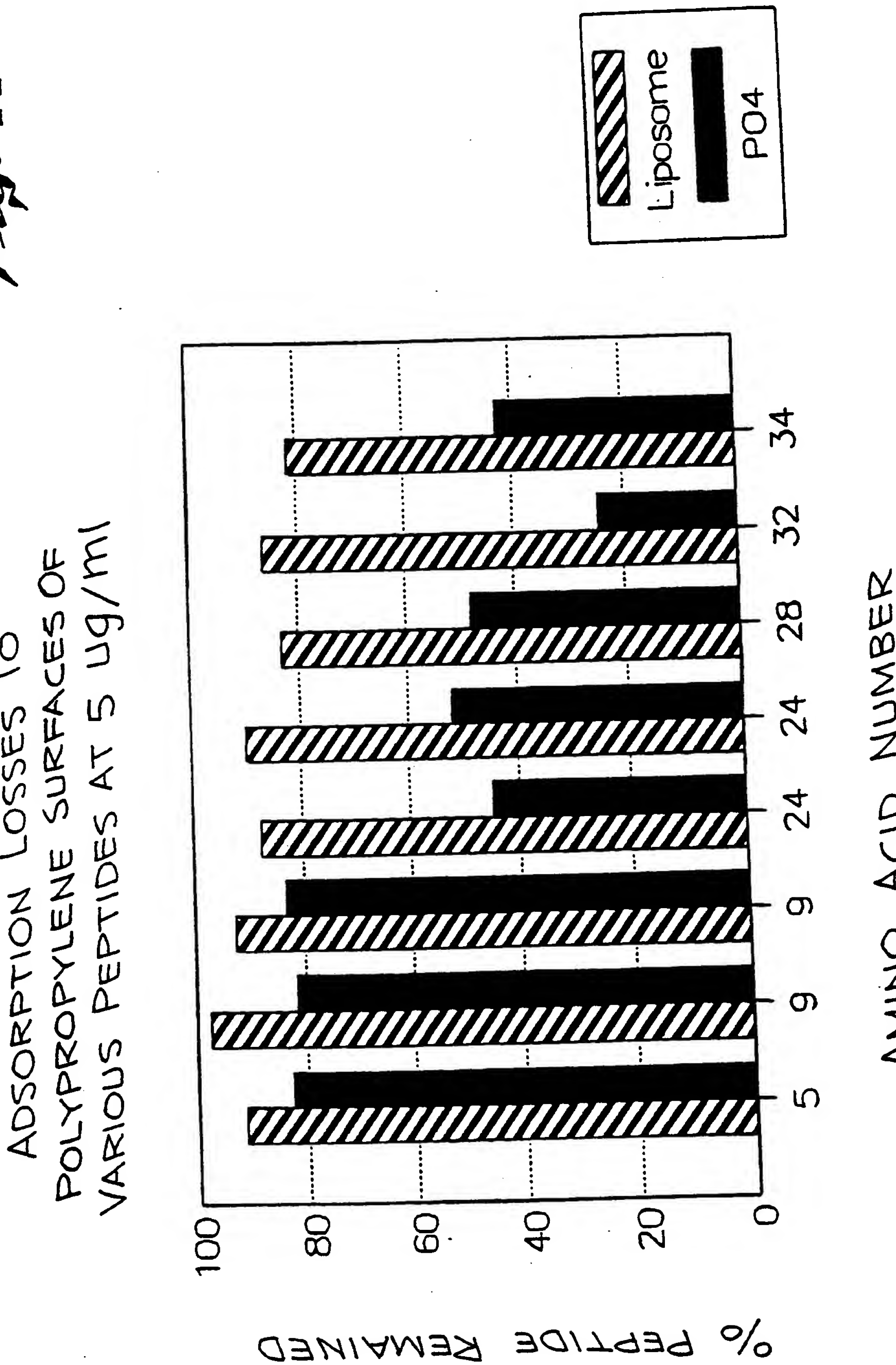

ADSORPTION LOSSES TO
POLYPROPYLENE SURFACES OF
VARIOUS PEPTIDES AT 5 ug/ml
Liposome
PO4
% PEPTIDE REMAINED
100
80
60
40
20
0
5
9
9
24
24
28
32
34
AMINO ACID NUMBER

Fig. 15.

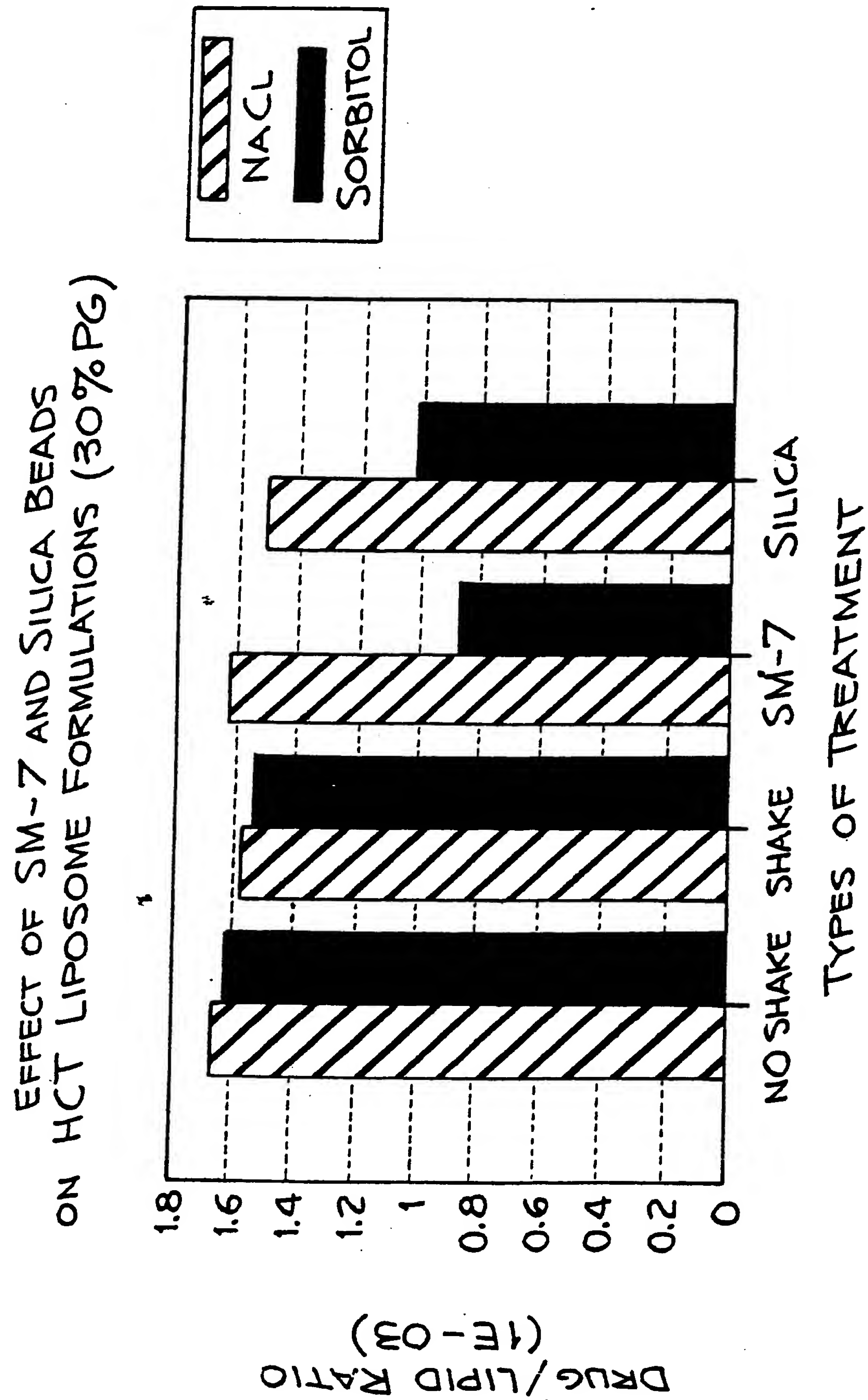

EFFECT OF SM-7 AND SILICA BEADS
ON HCT LIPOSOME FORMULATIONS (30% PG)
NACL
SORBITOL
DRUG/LIPID RATIO
(1E-03)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
NO SHAKE
SHAKE
SM-7
SILICA
TYPES OF TREATMENT

Fig. 16.

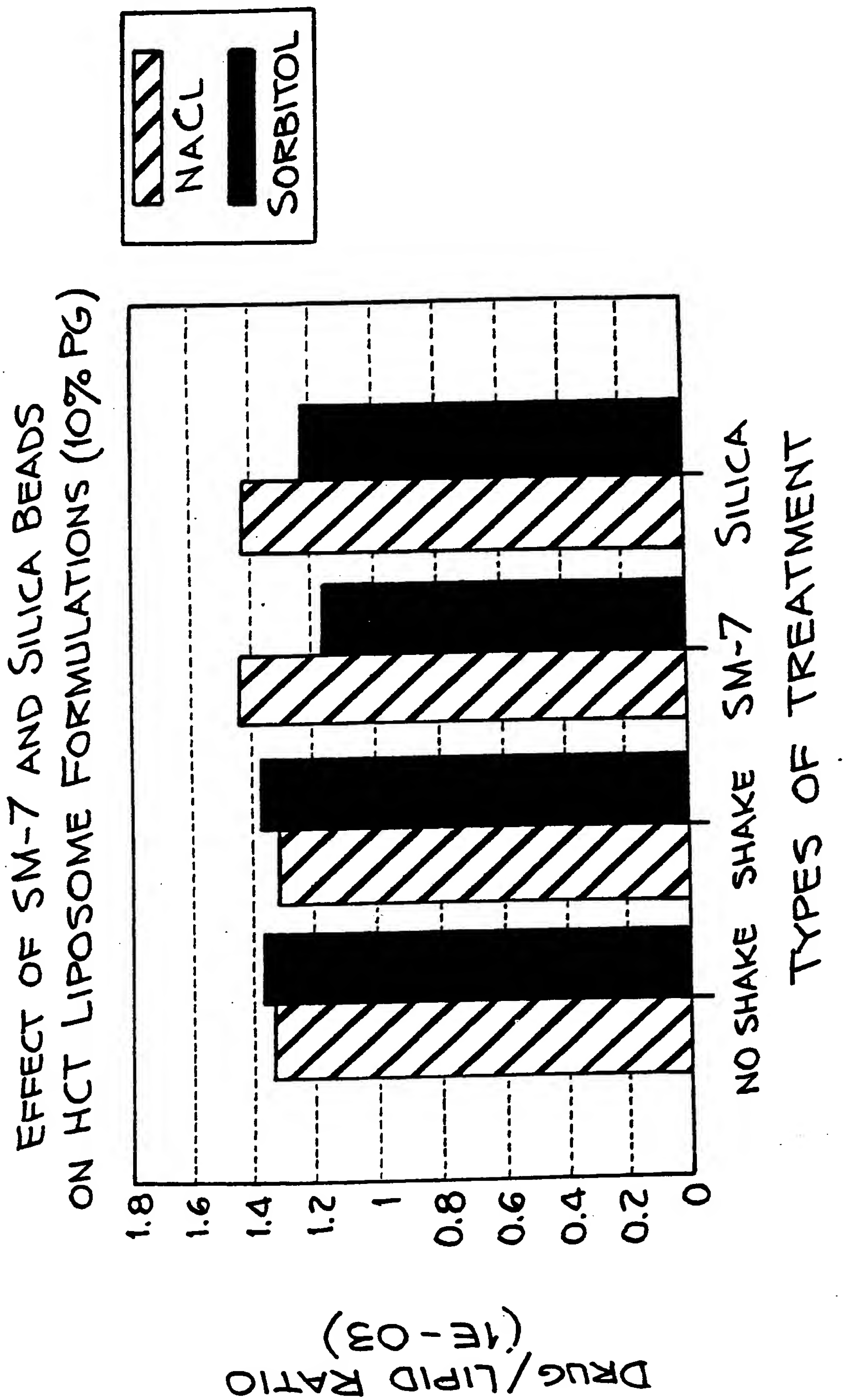
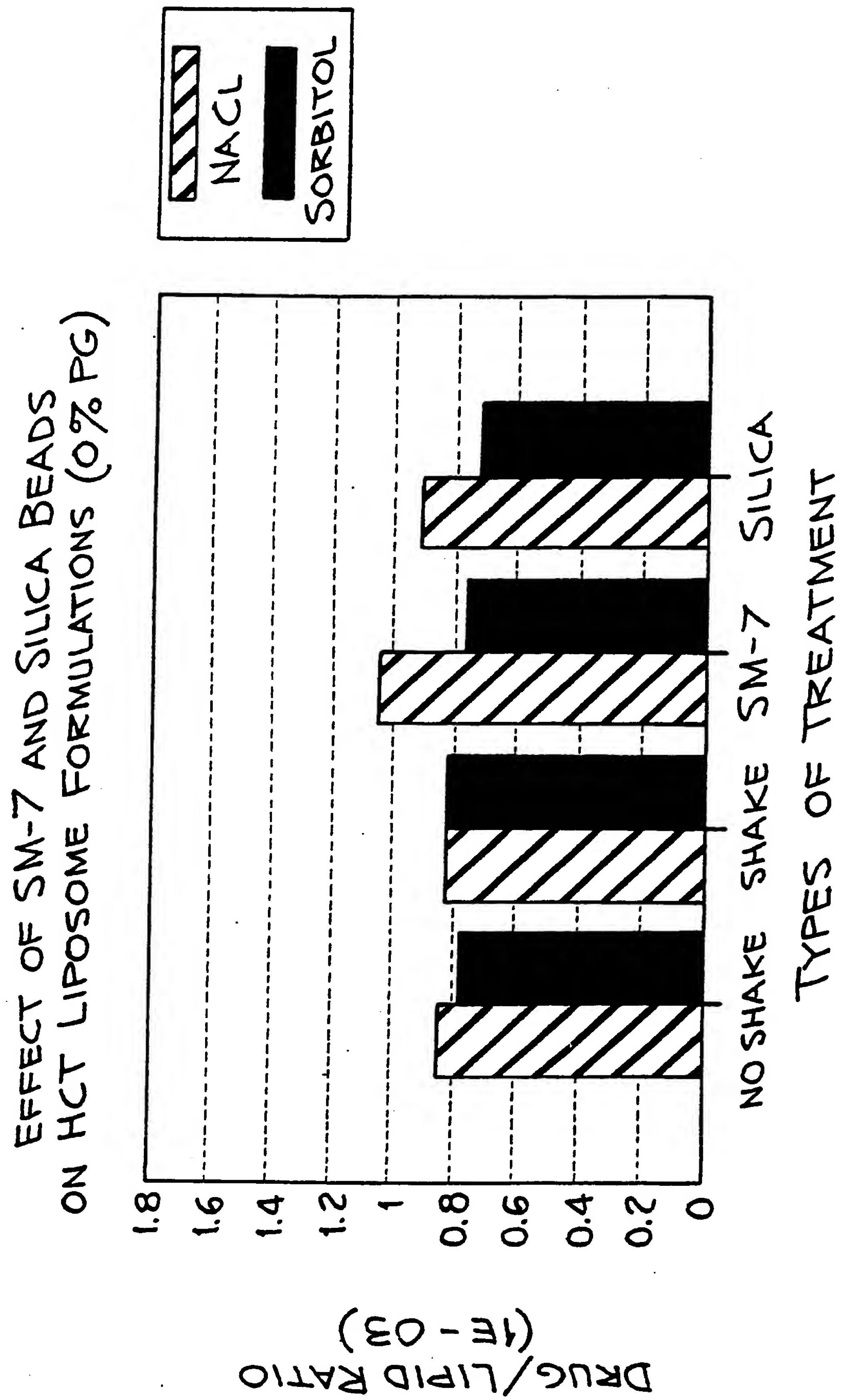

EFFECT OF SM-7 AND SILICA BEADS
ON HCT LIPOSOME FORMULATIONS (10% PG)
NACL
SORBITOL
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
DRUG/LIPID RATIO
(1E-03)
NO SHAKE
SHAKE
SM-7
SILICA
TYPES OF TREATMENT

Fig. 17.


EFFECT OF SM-7 AND SILICA BEADS
ON HCT LIPOSOME FORMULATIONS (0% PG)
NACL
SORBITOL
DRUG/LIPID RATIO
(1E - 03)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
NO SHAKE
SHAKE
SM-7
SILICA
TYPES OF TREATMENT

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/05163

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): A61K 9/50
U.S. CL. 424/450

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	424/450

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	US, A, 4,692,433 (HOSTETLER) 08 SEPTEMBER 1987; See columns 7 and 8.	1-43
Y,P	US, A, 4,895,719 (RADHAKRISHNAN) 23 JANUARY 1990; See columns 3, 7, 8, 19 and 23.	1-43
Y	US, A, 4,812,312 (LOPEZ-BERESTEIN) 14 MARCH 1989; See columns 2, 5 and 7.	1-43
Y,P	US, A, 4,913,902 (KILPATRICK) 03 APRIL 1990 See the entire document.	1-43

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

15 OCTOBER 1990

Date of Mailing of this International Search Report

18 JAN 1991

International Searching Authority

ISA/US

Signature of Authorized Officer

Demetra Mills

Demetra Mills